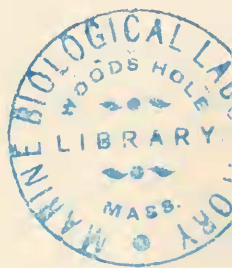


BIOLOGICAL BULLETIN



THE DEVELOPMENT OF THE SPERMATOZOÖN IN *CAVIA COBAYA*.¹

MARY T. HARMAN AND FRANK P. ROOT.

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INTRODUCTION.

The development of the spermatozoön in the Mammalia has been observed in a number of forms but a detailed study has been made in only a few instances. Among the workers who have published observations on the development of the mammalian spermatozoön are: Lenhossek (1898), Meves (1898), Benda (1897, 1906), Korff (1902), Duesberg (1908, 1920), Jordan (1911), Oliver (1913), Stockard and Papanicolaou (1918), Gatenby and Woodger (1921). There is a general agreement in the plan of the development but many differences of opinion exist with reference to the detail. Many of these differences are significant not only from the development of the spermatozoön itself but also from their bearing upon other biological problems. Since mammals are bisexual and have not been known to reproduce parthenogenetically, the continuity of the different parts of the male germ

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cell is of as much significance as that of the female germ cell. The loss of a part of the nucleus or even a part of the cytoplasm in the process of transformation of the spermatid into a spermatozoön may affect the theory of the vehicle of the bearers of the hereditary characteristics.

Cavia cobaya has been used as a subject of investigation for the development of the spermatozoön as often as any other mammal and the work has been done in as much detail and yet there is a lack of agreement upon a number of points. All authors are agreed that the spermatid is a typical one, similar to that described for insects and other animals and that the mature spermatozoön is composed of at least three parts or regions, the head, the mid piece and the tail. Also a fourth region, the neck, has been described by many workers. What parts of the spermatid contribute to the formation of each of these regions, of what each region is composed and whether or not the entire cell is used in the formation of the spermatozoön are questions upon which there are significant differences of opinion.

In our study of the development of the spermatozoön of *Cavia cobaya* certain things have been impressed upon us as being decidedly different from the observations of other authors. Of these we shall mention five as the most outstanding: (1) Following the last maturation divisions the chromatin material goes through an abortive preparation for division before there is much change in the shape of the cell. (2) We have found no loss of cytoplasm or sloughing off as has been described by many authors. It is true that we find stages when the entire developing spermatozoön is smaller than in previous stages but this seems to be due to a condensation of the material rather than a sloughing off of any part of it. This will be discussed in some detail in the body of the paper. (3) We have not found in any stage a filament extending out from the cytoplasm. We have diligently looked for it because we were very anxious to see the nature of this development and at what particular time it was first evident. In all of our observations the axial filament tapers to a blunt point at the terminis. There is no naked end filament even in the fully formed spermatozoön. (4) The tail is made up of three segments which are not only shown by the morphological structure but also by the

points of breaking as found in hundreds of broken specimens. (5) As was mentioned in our previous paper, the area of actively dividing cells are elliptical with the greatest diameter of the ellipse lengthwise of the tubule. Within this area the cells are generally in the same stage of development and only occasionally a stray cell is in some other stage.

It has not been our purpose to describe the origin of the cytoplasmic structures nor to say much about the confused nomenclature of the same. This has been only incidental to our purpose and we have discussed them only in so far as they contribute to the development of the spermatozoön. We have used much of the nomenclature of Bowen when it seemed applicable to our needs.

MATERIAL AND METHODS.

The material used is the same used in our previous paper (Herman and Root, 1926). In that paper will be found a detailed description of the fixing and staining of the material. All drawings have been made with the aid of a camera lucida and the magnifications are given in the description of the plates. With one exception, our drawings could be duplicated from hundreds of cells in our material. We make this statement to emphasize the fact that what we are showing is universal and not an exception which might be attributed to technique. The exception is the bent rod-shaped cytoplasmic inclusion in Fig. 7 which we have called a Golgi body.

(a) *Description of Material.*—We have begun with the changes which take place in the cell after the last maturation division has been completed. This is where we stopped in our last paper. For convenience of description these changes may be divided into three periods as follows: (1) The period with little change in the shape of the cell; (2) the period of elongation and (3) histogenesis of the elongated cell.

1. *The Period with Little Change in the Shape of the Cell.*—Significant changes take place both in the nucleus and the cytoplasm before there is much change in the shape of the cell. At the end of the last maturation division the chromatin passes through a typical telophase. It becomes finely granular and a definite nuclear membrane is formed. Following this there takes place what

we have chosen to call an abortive attempt to divide again. The chromatin forms into a close network having irregular clumps and the nuclear membrane nearly disappears, Fig. 1. Then the nucleus increases in size and the chromatin material is in a more nearly continuous spireme, Fig. 2. The chromatin clumps become more numerous and prominent. These changes continue until a compact unbroken spireme is formed, Fig. 3. Then there is an attempt to form chromosomes, Fig. 4. The chromatin knots are numerous and the spireme has been separated into irregular pieces which may be compared to chromosomes but which lack the smooth contour and the compact appearance of chromosomes. We have called these masses of chromatin material "chromatin knots." There remains some trace of the spireme but it is little more than a suggestion. Following this the chromatin knots become more granular and there is no further indication of a division of the cell, Figs. 5 and 6. Now the entire cell begins to contract and to become compact. At first this is more evident in the nucleus than in the cell body. The chromatin material becomes finely granular and only traces of the spireme are discernible. The entire nucleus occupies much less space, Figs. 7, 8, and 9.

While these changes have been taking place in the nucleus, changes have been occurring in the cytoplasm. A number of spherical bodies varying in size appear in the early spermatid. These are the Golgi bodies. There is a lack of constancy in the number and the size of these Golgi bodies. They are found in the periphery of the cell as well as near the nucleus. Sometimes they may indent the nuclear wall, Fig. 2. With Heidenhain's haematoxylin they are stained like chromatin which emphasizes their spherical form and distinguishes them from the surrounding cytoplasm in the early spermatids. They are finely granular like the surrounding cytoplasm but the granules are more closely compact than in the other parts of the cytoplasm. Each Golgi body has the appearance of a sphere surrounded by a halo.

An idiosome is always near the nucleus. In section it is crescentic in shape, with the concave side toward the nucleus, and stains like the surrounding cytoplasm from which it is distinguished by its more homogeneous structure. In older stages it comes to lie in contact with the nucleus then there is a more defi-

nite orientation in its position than that of the Golgi bodies and the nucleus. At least one Golgi body is always near the nucleus and at the same time near the idiosome, Figs. 1, 3, 4, and 5. Some of the Golgi bodies form a group near the nucleus on the side opposite to the idiosome.

The idiosome becomes closely applied to one side of the nucleus, Figs. 8 and 9. The idiosphere is in the concavity of the idiosome between it and the nucleus, *NE* in Figs. 8 and 9. The entire cell, both cytoplasm and nucleus, has become smaller and there is evidence of the beginning of the change in the shape of the cell.

2. *Period of Elongation.*—With the diminution of the volume of the cell there is the beginning of an elongation in the axis determined by the idiosome, on the one side, and the Golgi remnant on the other. This elongation occurs in the entire cell affecting the shape of both the cytoplasm and the nucleus, Figs. 10 to 22. During this time the cell is in intimate connection with the Sertoli cell which is at first small but later increases enormously in size. Fig. 20 illustrates a Sertoli cell with some of the associated spermatids in an elongated form. The part of the spermatid destined to become the head is directed toward the base of the Sertoli cell and the other part toward the lumen of the tubule. This is true regardless of the stage of development. When the spermatozoa are freed from the Sertoli cell they are not in a mixed up mass but are in bundles lying almost parallel, with most of the heads in the same direction. This could easily be accounted for by the fact that they have a definite orientation during their development. While the cell is elongating the idiosphere becomes embedded in the idiosome. The idiosphere stains more densely than the idiosome. Thus the idiosome has the appearance of having a core. The idiosome and the idiosphere form an elongate body pointed at its distal end and truncate at its proximal end, Figs. 13 to 17. Fig. 14 is a surface view while the other figures show the idiosphere surrounded by the idiosome. The idiosome and the idiosphere may now be called the acroblast.

The chromatin material in this stage has become finely granular and can scarcely be distinguished from the cytoplasm either by its staining reaction or by its structure. The nucleus elongates

until it becomes cylindrical, Figs. 14 to 17. A thin coating of cytoplasm surrounds it and extends in the direction opposite to the acroblast. Later the nuclear material takes a position to one side of the cylindrical mass and the cytoplasm forms a flattened area to the other side extending from the acroblast to the other end of the cell, Figs. 18 and 19. In these figures the acroblast is becoming rounded and is beginning to take a position to the side of the nucleus instead of completely anterior to it as in the earlier stages. The nuclear material is beginning to become more condensed and is spread out over a wider surface. Posterior to the nucleus there are three fine thread-like filaments which spread into a somewhat fan-shaped mass in the surrounding cytoplasm. Associated with these filaments are two areas of cytoplasmic granules. One area is at the extremity of the filaments and the other area is near the base of the nucleus, Figs. 19 and 21. Following this stage, the cytoplasm which is transforming into the tail of the spermatozoön condenses rapidly and becomes very elongate.

3. *Histogenesis of the Elongate Cell*.—In the histogenesis of the elongate cell the three regions usually recognized in a mammalian spermatozoön begin to be evident. At first the nucleus and the acrosome which make up the head are much longer than they are wide and become cylindrical and somewhat enlarged at the free end. The nucleus is now at one side of the cytoplasmic acrosome and it does not extend entirely to the free end of the developing spermatozoön. The mid-piece which is occupied largely by the spiral filament in the adult spermatozoön becomes granular in regularly arranged clumps, *SF*, Fig. 23. This is the region which was occupied by the three thread-like filaments in Figs. 19, 21, and 22. One of the most noticeable changes is in the tail region. There is a very rapid condensation of the cytoplasm which was spread out in a fan-shaped mass to a tapering whip-like flagellum. The tail is composed of three segments. The first one is about as long as the mid-piece, the second one in the early stages is about the same length and the third or terminal one is a little longer than the combined length of the other two. It gradually tapers to a blunt point. We have not found in any stage of development any unsheathed terminal filament.

As differentiation progresses there is a greater difference be-

tween the sizes of segments one and two of the tail. The second segment elongates more than the first and tapers more as it increases in length. The segments are recognized by distinct markings and when the tails of the spermatozoa are broken off, the break is always at the union of two of these segments. Seldom is the tail broken from the head at the anterior part of the mid-piece and practically never is the tail broken off at the posterior part of the mid-piece. A few of the tails are broken at the end of the first segment. Most frequently the break is at the distal end of the first segment, less frequently between the second and the third segments. We never find the tail broken within any segment.

Figures 24, 25, and 26 are illustrations of a mature spermatozoön viewed from different positions. The acrosome forms a hood-shaped covering to one side and anterior to the nucleus. The head is broad from side to side, Figs. 24 and 25, but rather thin when seen from the edge, Fig. 26. The regularly arranged clumps of cytoplasm in the mid-piece, mentioned above, develop into a distinct spiral, with the coil always counter-clockwise from the anterior part of the mid-piece. The last two coils are almost rings and might be termed, annulus. There is no annulus separate from the spiral filament. As is shown in the drawings the coils are not always regular. They remind one of a spring that has been put at a tension and the rebound has not been the same in all regions of the spring. The first four coils of the spiral filament are inclosed by a thin bladder of cytoplasm.

DISCUSSION.

In the transformation of the spermatid into the spermatozoön little attention has been given to the behavior of the chromatin material other than it finally becomes condensed into a more or less homogeneous mass which appears solid and is stained heavily with nuclear dyes. Meves (1899) has shown the nuclear material formed into clumps before there has been much change in the shape of the spermatid. Ballowitz (1891) has also this clumping of the chromatin material in his drawings. Neither author has discussed this change nor has mentioned further changes in the chromatin. They state that the nucleus forms the greater part of the head of the spermatozoön.

In one of the Hemiptera, Bowen (1920) says that "the head undergoes a characteristic change resulting in what appears to be a complete vacuolization of the chromatin lining. Then the chromatin collapses toward the axis of the head, etc."

We have shown that after the last maturation division the chromatin material passes through changes which are similar to those in a cell that is getting ready to divide until there is the breaking up of the chromatin material into clumps. A significant difference, however, between these changes and the changes previous to the maturation divisions is that there is no synzeisis and no double thread. We raise the question whether these changes influence the behavior of the cytoplasm in the process of transformation and thus the attempt at division is aborted or whether the changes in the cytoplasm arrest the changes taking place in the nucleus.

The small size of the spermatozoön in comparison with the early spermatid is recognized by many authors. Some of this difference in size has been accounted for by a loss in cytoplasm. In the formation of the spermatozoön of vertebrates, Kölliker as early as 1856 and la Vallette St. George (1865) described the "sloughing off" of the cytoplasm. Later Biondi (1885), Benda (1897), Hermann (1889), and Neissing (1889 and 1896) agree that there is a loss in cytoplasm by a sloughing off. Meves and Ballowitz have shown cytoplasm loosely connected with the transforming tail part.

This difference in the size of the spermatozoön and the spermatid is recognized in the insects. Montgomery (1911) states that in *Euschistus* "no evidence was found for the casting off of any substance by the sperm."

In the formation of the spermatozoön in *Paratettix*, Harman (1915) did not find any loss of cytoplasm. The cytoplasm condensed around the axial filament but there was no indication of a sloughing off either in the appearance of the cell or the remains in the follicle.

In our material, the spermatozoön is greatly reduced in size during the process of transformation, but we have found no evidence in any region of a loss of material. We have shown, Figs. 24, 25 and 26, that a bladder-like structure of cytoplasm is pres-

ent in the transformation but that this condenses around a portion of the middle piece and there is no evidence that it is sloughed off.

Most authors recognize that the greater part of the head of the spermatozoön is formed from the nucleus of the spermatid and furthermore, they recognize that this head is much smaller than the original nucleus. No one has described the loss of nuclear material. This agrees with our observations. We believe that this diminution in size is due to a condensation in which the material appears more compact than in earlier stages.

Meves (1899), Ballowitz (1891), and Duesberg (1910) show a thread-like filament extending out from the cytoplasm in the very early stages of development. Meves describes this filament as arising from one of the centrosomes which gives rise to the posterior nodule and this filament which in turn becomes the axial filament. He represents the distal end of this filament as remaining unsheathed and forming the terminal filament. We have found no unsheathed filament at any stage of development. We have shown, Figs. 18, 19, 21 and 22, three filamentous structures which lie deep in the cytoplasm. These filaments are spread out distally into a fan-shape. Associated with these structures are two areas of granules. We have not traced the detailed history of these granules but we have noted that they finally become enclosed in the cytoplasm which rapidly condenses and with the associated filaments form the tail of the spermatozoön. There is a gradual tapering of the tail to a blunt point. This tapering takes place in the axial filament as well as in the sheath which encloses it entirely to the distal end.

The tail is made up of three segments as we have shown in Figs. 23, 24, 25, and 26. Early in our study of the mature spermatozoön, among mutilated specimens we were impressed with the regularity of the lengths of the pieces of the tails. These lengths were quite constant whether the spermatozoa were in bundles, merely a few together or even if a single spermatozoön was broken. The pieces were in three different lengths which corresponded to the three segments of the tail. Measurements showed only a slight variation. It would seem that the tail is weaker at the points of junctions of the segments.

The transformation of the spermatid into a spermatozoön takes place in definite areas which are elliptical in shape. The greatest diameter of the ellipse is always lengthwise of the seminiferous tubule and the shortest diameter never exceeds two-thirds of the circumference of the tubule.

SUMMARY.

1. The transformation of the spermatid into a spermatozoön takes place while the spermatid is closely associated with a Sertoli cell and it does not become free in the lumen of the seminiferous tubule until the spermatozoön is matured.

2. In the early stages of transformation the cell goes through a growth period in which the entire cell gets larger and the chromatin material goes through an abortive preparation as if for division.

3. During the period of elongation there is a reduction in the volume of the cell and a rearrangement of its parts.

4. No "sloughing off" or loss of cytoplasm has been observed.

5. The head of the spermatozoön is composed of two parts, the head proper which arises from the nucleus and the head cap or acrosome which arises from the idiosome and the idiosphere.

6. There is a cytoplasmic bladder-like structure around the anterior part of the mid-piece.

7. The tail is composed of three segments terminating in definite nodes.

8. We find no indication of an unsheathed terminal filament either during the transformation or in the mature spermatozoön.

9. The tails of the spermatozoa are always toward the lumen of the seminiferous tubule.

10. The areas of transformation are elliptical in shape with the long axis of the ellipse corresponding to the length of the seminiferous tubule and the short diameter of the ellipse never exceeds two thirds the circumference of the tubule.

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EXPLANATION OF PLATES.

All the drawings were made with the aid of a camera lucida, a 1.9 oil-immersion objective and a number 6 compensating ocular at table level. Figure 20 was enlarged two diameters. All other drawings were enlarged four and one half diameters. The reproductions were reduced one half from the original.

PLATE I.

FIG. 1. Spermatid soon after the last maturation division. *G*, Golgi bodies; *N*, nucleus; *I*, idiosome.

FIG. 2. Spermatid showing increased size. *G*, Golgi body.

FIG. 3. Spermatid with the chromatin in the form of a spireme. *I*, idiosome; *G*, Golgi body.

FIGS. 4, 5, AND 6. Spermatid showing an abortive attempt to form chromosomes. *I*, idiosome; *G*, Golgi body.

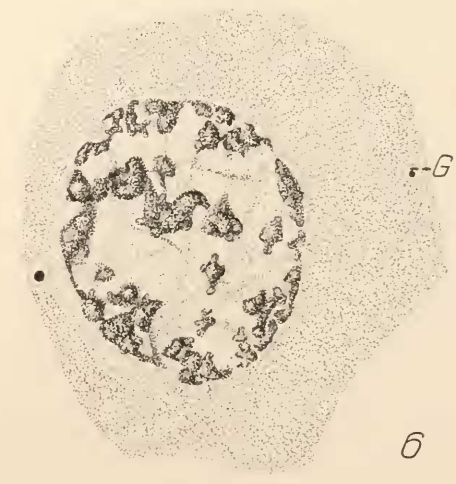
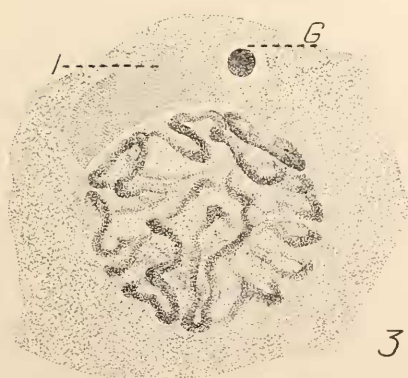
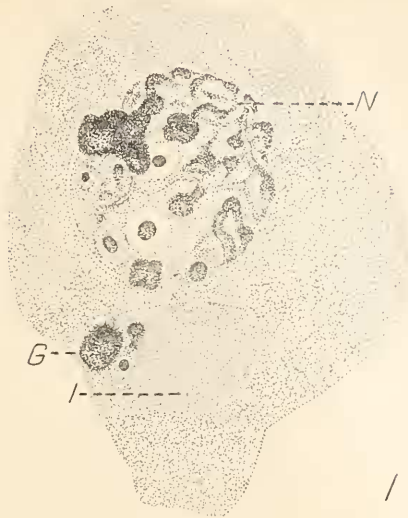


PLATE II.

FIG. 7. Spermatid showing the chromatin finely granular and the beginning of the contracting of the entire cell.

FIG. 8. Spermatid showing the idiosome closely applied to the nucleus and the appearance of the idiosphere. *I*, idiosome; *NE*, idiosphere; *G*, Golgi body.

FIGS. 9 AND 10. Spermatids showing a great reduction in size. *I*, idiosome; *NE*, idiosphere.

FIGS. 11 AND 12. Spermatid showing the ovoid shape which is the beginning of the elongation. *I*, idiosome; *NE*, idiosphere; *G*, Golgi body; *C*, cytoplasm.

FIGS. 13 AND 14. Spermatid showing the beginning of the elongation of the idiosome and the idiosphere. *I*, idiosome; *NE*, idiosphere; *N*, nucleus; *C*, cytoplasm; *G*, Golgi body; *A*, acroblast.

FIGS. 15, 16 AND 17. Spermatids showing the elongation of the nucleus and the spreading out of the cytoplasm in a fan-shape. *A*, acroblast, *N*, nucleus; *C*, cytoplasm.

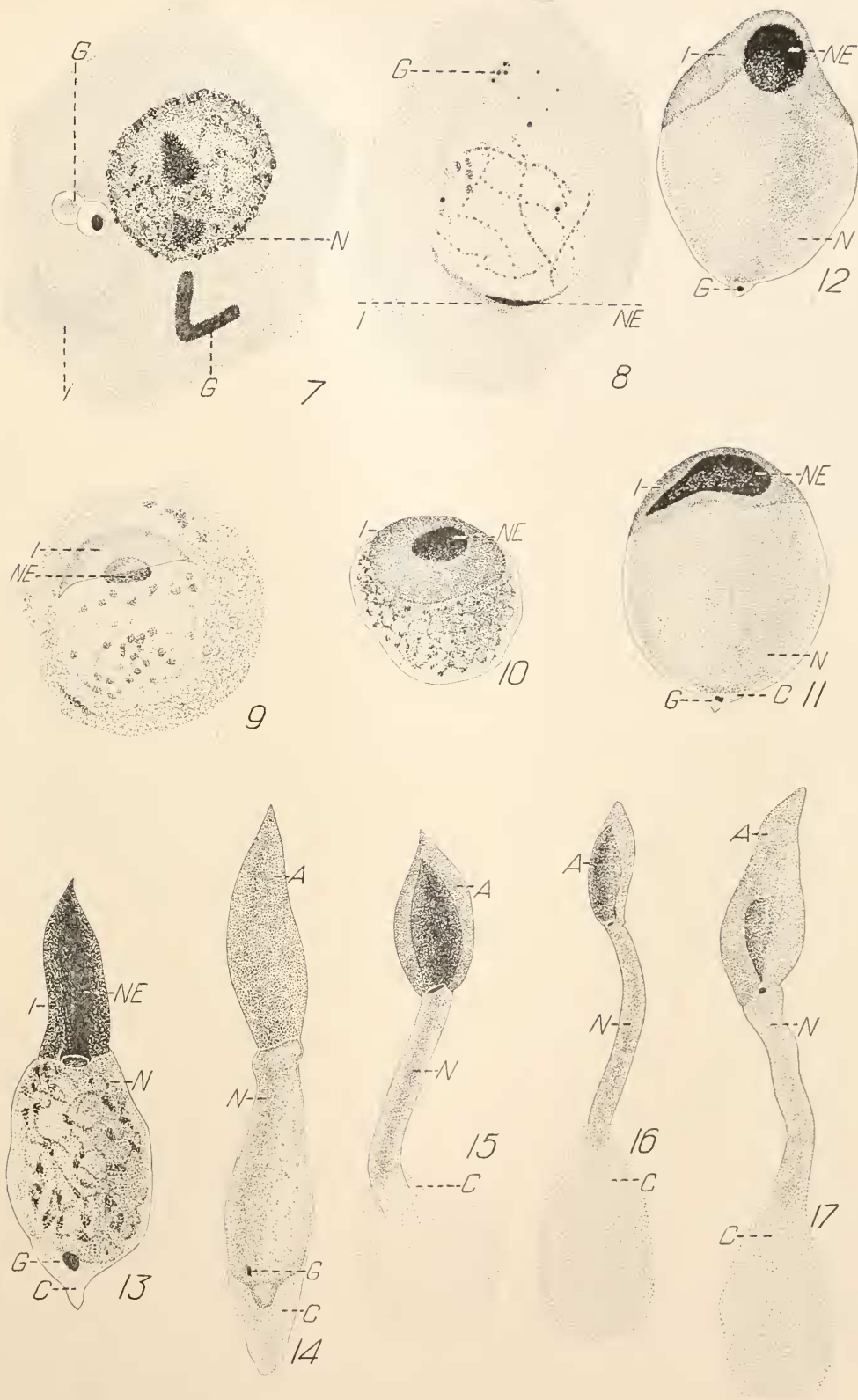


PLATE III.

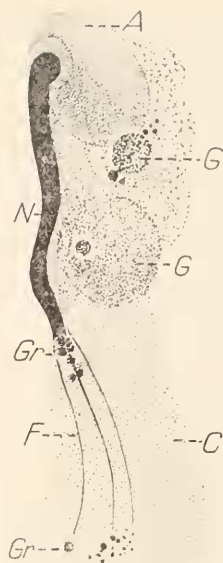
FIGS. 18 AND 19. Spermatids showing appearance of filaments from the nucleus and the extension of the acroblast to the side of the elongated nucleus. *A*, acroblast; *N*, nucleus; *C*, cytoplasm; *F*, filaments; *G*, Golgi body; *Gr*, granules.

FIG. 20. Sertoli cell with some of the associated spermatids in an elongated form. *S*, spermatids; *N*, nucleus; *AC*, spermatogonial cell.

FIGS. 21 AND 22. Spermatids, a continuation of the development shown in Figs. 18 and 19. *A*, acroblast; *N*, nucleus; *F*, filaments; *C*, cytoplasm; *Gr.*, granules.



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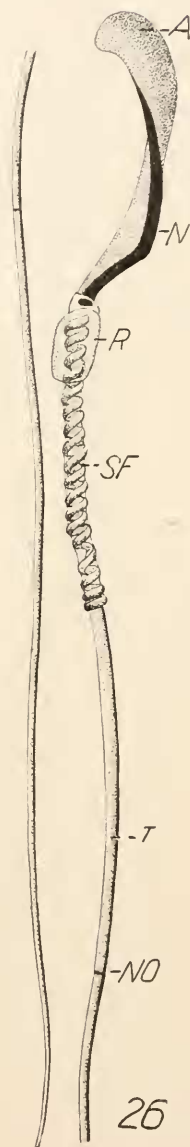
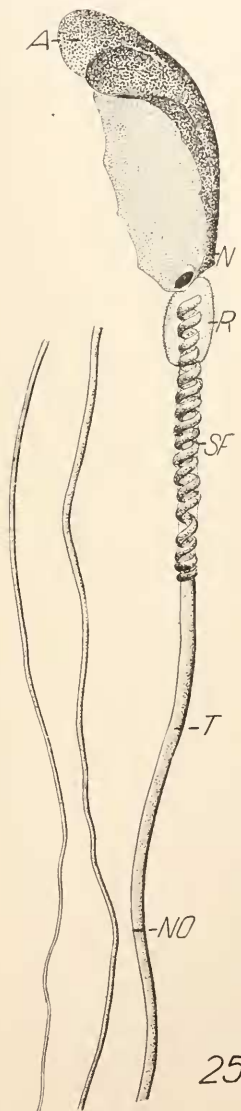
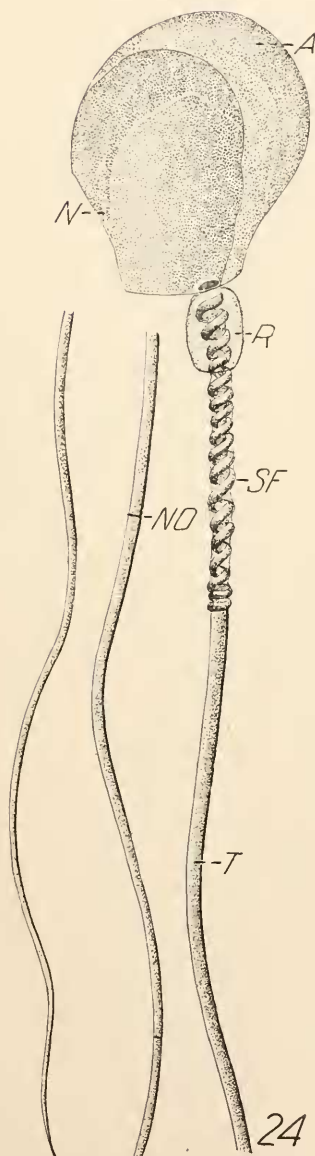
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PLATE IV.

FIG. 23. Spermatid almost transformed, viewed from one edge. *N*, nucleus; *SF*, spiral filament in formation; *A*, acrosome; *LT*, tail segment; *NO*, node.

FIGS. 24, 25, AND 26. Mature spermatozoa. Fig. 24 viewed from convex surface, Fig. 25 from side angle and Fig. 26 from edge of head. *A*, acrosome; *N*, nucleus; *R*, residual cytoplasm; *SF*, spiral filament; *T*, tail; *NO*, node.



STUDIES ON THE SECONDARY SEX CHARACTERS
OF CRAYFISHES. VIII. MODIFIED THIRD
ABDOMINAL APPENDAGES IN MALES
OF *CAMBARUS VIRILIS*.

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The first and second abdominal appendages of males are habitually modified in *Cambarus* for the purpose of copulation. The individual parts of the first pair of appendages are fused and twisted and lie compactly in a groove on the ventral side of the thorax. The appendages of the second abdominal segment resemble the typical swimmeret in general plan (Figs. 1 and 6). However, the protopodite is elongated and heavier, the basal unsegmented portion of the endopodite is likewise reinforced, bearing a conspicuous triangular shoulder. The terminal segmented portion of the endopodite is much reduced. The remaining swimmerets are unusually quite typical.

There is apparently only one published record of a modified third abdominal appendage. Moenkhaus, Proceedings of the Indiana Academy of Science, 1903, pp. 111 and 112, describes a specimen of *Cambarus virilis* bearing such a modification as follows: "The first and second pairs of appendages were modified in the usual way and in no way differed from corresponding appendages in the normal male of the same species. The additionally modified third pair resemble in plan almost exactly the second pair. The exopod and the segmented flabellum of the endopod are much less reduced and much more extensively provided with feathered setae than the second pair. They are about the same size and in position converge and fit against the second pair of appendages much in the same manner that these do against the first. Whether they were in any way functional I am, of course, unable to say." Another specimen with a modification similar to but not so fully developed as the one described by Moenkhaus was collected by Dr. H. J. Van Cleave of the University of Illinois and appears in his collection.

Since crayfishes are in such common use as laboratory subjects it seems likely that any considerable occurrence of this aberrancy would have been noted and described. The writer has examined thousands of crayfishes during the past seven years, always with the object of finding peculiarities in the secondary sex characters and while large numbers of specimens have been found in which other aberrancies occurred, not one was found with this type of peculiarity until the lot described came to light.

A collection of several hundred specimens which had been taken from the Fox River between Green Bay and DePere, Wisconsin, during the summer of 1927, was being used in the Zoölogy Laboratory at Northwestern University. A specimen having peculiar appendages was discovered by chance and the writer then examined the entire lot. Forty-six of a total of three hundred and forty-two males were found which had third abdominal appendages modified somewhat like those of the second abdominal appendages. No other peculiarities were noted among the males, but one female in seventy possessed a pair of copulatory hooks on the third walking legs like those of the male. The latter type of aberrancy is the most common and it is surprising to find a type that is apparently rare in greatly superior numbers.

DESCRIPTION OF SPECIMENS.

The male specimens with the modified third abdominal appendages are about thirteen and a half per cent. of the total number examined. They range in length from 79 to 107 mm. Twenty-eight are second form and eighteen are first form males. A fairly complete series is represented in the aberrant appendages. In some, the third abdominal appendages varies only in the presence of a slight projection upon the inner border of the endopodite between the basal unsegmented and the terminal segmented portions (Figs. 2 and 3), while at the other end of the series the modifications are practically like those of the second abdominal appendages (Figs. 4 and 5). There is apparently no relation between the extent of modifications of the appendages and the size of the animals. In form I. specimens the angles upon the shoulder of the aberrant appendages are sharper and stronger than those of form II., but this might have been expected

since the same is true of the usual modified appendages in normal form I. and form II. males.

The first and second abdominal appendages are normal in every respect in all the specimens.

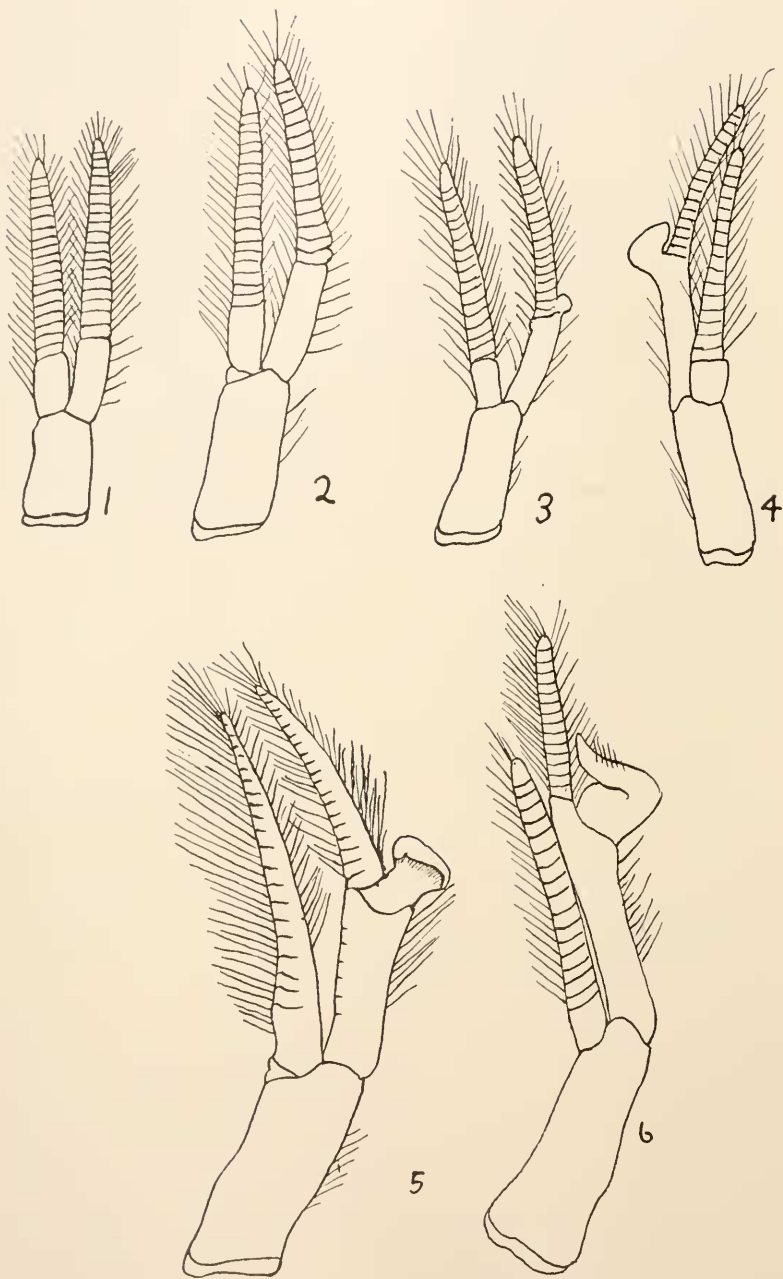
DISCUSSION.

In attempting to find an explanation for the large occurrence of a rare aberrancy, age, accidental embryonic development, effect of environment or peculiar genetic constitution might be suggested at first thought as causal factors. The fact already noted in this description that size, and therefore age, and degree of development of the peculiarities in the appendages are independent would seem to eliminate age as a factor. Accident might be called upon to account for a specimen or two but scarcely for so large a number as is represented here. It has yet to be shown that environment has played any part in the development of the secondary sex characters of crayfishes, nor indeed, in modifying them.

Peculiar genetic constitution seems to be the logical factor here. It has already been shown for other aberrant conditions in sex characters of crayfishes that there is a strong tendency for the development of one type of peculiarity in one locality and the present case is another instance of the same tendency. It has been argued in these other instances that the peculiarity might easily arise and perpetuate itself as a mutation and the explanation is again offered for the case in hand.

It does not seem likely that this modification has any functional significance. Specimens more radically modified in other sex characters have been functioning normally and there is no reason to believe that this slight peculiarity would make males any more efficient nor that it would interfere with copulation.

The series offered in the specimens here, from the slightly modified to the most completely modified may give a clue as to the evolutionary changes through which the normal second abdominal appendages came in the course of their development. This is speculative, of course, but we have here an actual series ranging from a practically unmodified third abdominal appendage to one which almost exactly duplicates the normal second. Unless the highly peculiar second abdominal appendages arose with all their



peculiarities fully formed in one stage it is easy to believe that they arose through a series of changes such as is represented here. The first stage would be represented by the development of a low projection on the inner surface of the endopodite between the unsegmented basal portion and the segmented terminal portion. Subsequent changes would involve an enlargement of this spur and a molding of it until it had assumed the shape found in the normal second appendage of the male. Other changes would involve an elongation and an enlargement of the propodite, and an enlargement and a strengthening of the basal portion of the endopodite together with a reduction of the terminal segmented portion of the endopodite.

In aberrant females having first abdominal appendages modified like those of males the second abdominal appendages are also sometimes modified. Such aberrant females are rare but even in a small number various degrees of modification are shown in the second appendages. These second abdominal appendages are identical in their structural peculiarities with the third abdominal appendages described here and are similar also in that they show various stages of development.

EXPLANATION OF FIGURES.

Note: All figures are drawn to the same scale.

FIG. 1. Unmodified left third abdominal appendage of normal male.

FIG. 2. Left third abdominal appendage of aberrant male measuring 88 mm. Callosity on endopodite is showing first stage of development.

FIG. 3. Left third abdominal appendage of aberrant male measuring 82 mm. Callosity on endopodite much larger.

FIG. 4. Right third abdominal appendage of aberrant male measuring 80 mm. The propodite is longer, the basal portion of the endopodite elongated and the shoulder upon the endopodite is more prominent.

FIG. 5. Left third abdominal appendage of aberrant male 103 mm. in length. Modifications almost equal to those of the normal second abdominal appendage.

FIG. 6. Left second abdominal appendage of normal male measuring 98 mm.

NATURAL HISTORY OF SHIPWORM, *TEREDO NAVALIS*, AT WOODS HOLE, MASSACHUSETTS.

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From the Marine Biological Laboratory, Woods Hole, Mass.

SECTION I. OCCURRENCE.

The common species of shipworm at Woods Hole, as identified by Kofoed and Clapp, is *Teredo navalis*. The date of its first appearance in this region is not known. Verrill lists it in his "Invertebrate Animals of Vineyard Sound and Adjacent Waters" (1871). Whatever its history in American waters may have been, it is now known to occur throughout the entire North American coast from Alaska to Labrador.¹ The present study has been carried on during the past four years and in that time no other species has been collected. It is known, however, that *Bankia fimbriata* occurs in this region, although in comparatively small numbers. During the year this work was first undertaken it was difficult to obtain *Teredo* in sufficient numbers for satisfactory study, but this is not an indication that the species is not abundant in New England waters. The reason for an apparent scarcity is that shipworms are inaccessible, being, for the most part imbedded in piles and permanent structures. Subsequently, by putting out suitable timbers during one summer to be studied the next, it has been an easy matter to obtain *Teredo* in abundance. Lobster pots² and 2×4 stakes have been found to be the most convenient. If these timbers are exposed to the water during the latter part of the summer they are found to contain sexually mature worms by the beginning of the breeding season the following June. The 2×4 stakes give best results if exposed

¹ Nelson, '22, speaks of an infestation of *Teredo navalis* in Barnagat Bay, New Jersey, as a sudden outbreak. He is probably in error in thinking that this species arrived so recently on the New England coast.

² Lobster pots are constructed of small slats about the size of ordinary plasterer's lath, 2 in. broad and $\frac{1}{2}$ in. in thickness.

during July or early August, but the smaller timbers are liable to complete destruction before winter if put out early in the summer.

Teredo do not grow large in small timbers such as are used in the construction of lobster pots, but are easily removed from such small strips of wood, thereby facilitating study. The size attained depends upon the degree of crowding. To ascertain the size to which *Teredo* will grow, it is necessary to supply larger pieces of wood and 2×4 stakes are excellent for the purpose. With a drawing knife it is possible to expose the entire burrow in a few minutes because *Teredo* tunnels with the grain of the wood, usually within half an inch of the surface. A study of such stakes has shown that *Teredo* larvæ attack the wood in great numbers at the mud line but less and less abundantly from the bottom to the surface of the water. Three fourths of the *Teredo* burrows in an exposed timber occur within two or three feet of the mud line. Very few are found more than four feet above the bottom.

SECTION II. ANATOMY, PHYSIOLOGY AND BEHAVIOR.

The anatomy of *Teredo* has been accurately described by several early investigators and more recently the shell and digestive tract have received attention by Miller and Lazier, whose admirable work is published in four papers. It is sufficient here to say that the shipworm has the structure of an ordinary lamelli-branch in which the body is much elongated and in which the bivalve shell is highly modified in adaptation to the burrowing habit. In one particular my observations are not in agreement with those of Miller. He attributes the formation of the rings of growth, the rasping ridges, and denticles of the shell to alteration or fluctuation in the food supply which, according to his conception, results in corresponding periods of slow and rapid growth. This may account for the annual rings of growth of certain mollusks and has been so interpreted, but it could hardly account for the rings and ridges on the shell of this young animal which adds two rings per week in the early stages of its development. These sculpturings of the shell which adapt it to burrowing are undoubtedly due to the action of little tongues

of mantle tissue which are pushed up over the edge of the shell during deposition of the shell material. This process of shell sculpturing was observed in the large lamellibranch *Atrina rigida* (Grave, '09). The peculiar form and pattern of the shell is specific and is a matter of inheritance, but the building process is due to the peculiar manipulation of the mantle and not to alternate periods of starvation and plenty.

The physiology of digestion has been studied particularly in recent years by Dore, Miller and Potts.

Potts ('24) corroborates the work of Dore and Miller ('22) in showing that as the shipworm burrows through the wood it swallows the chips and derives some nourishment from them. A large section of the digestive tract seems to be devoted entirely to the digestion of wood (the cæcum and liver). Potts believes that wood is the only food of *Teredo* but Miller shows that the digestive tract contains diatoms as well as wood. The burrow mainly serves as a means of protection.

As the *Teredo* grows it enlarges its burrow proportionately until at maturity it may be 16 inches in length and have a diameter of $\frac{3}{8}$ of an inch (40×1 cm.). A pile or other exposed piece of timber may be honeycombed with *Teredo* tunnels without showing on the surface that it is infested. The only opening of the burrow leading to the outside is the minute pore through which the young *Teredo* entered the wood as a metamorphosing veliger. Although less than .35 mm. in diameter and therefore too small to be seen readily by the unaided eye, it is through this passage that the siphons are protruded to obtain respiratory currents and food other than wood. The shipworm feeds upon minute organisms derived from water currents that pass over its gills for respiration, just as in ordinary lamellibranchs. It is in fact an elongated lamellibranch, whose burrowing shell covers only its anterior tip, leaving most of the body and the siphons unprotected except for the wooden shell-lined burrow.

CHARACTER OF THE BURROW.

The burrows are always lined by a calcareous substance, except at the anterior end, where further excavation is taking place.

This shell-like material is secreted by the general surface of the body or mantle. It has been suggested that this lining of the burrow not only makes a smooth surface, but shuts out wood acids as well as external enemies which might otherwise injure the soft body of the animal. Even the outer pore-like opening is lined with this secretion and is divided transversely by a partition, so that the siphons protrude through two minute pores just large enough to transmit them. While the shipworm is not feeding, or when it is disturbed, the siphons are withdrawn and the external openings are plugged by two curious horny pallets, as they are called, situated one on each side of the siphonal region. See Figure I.

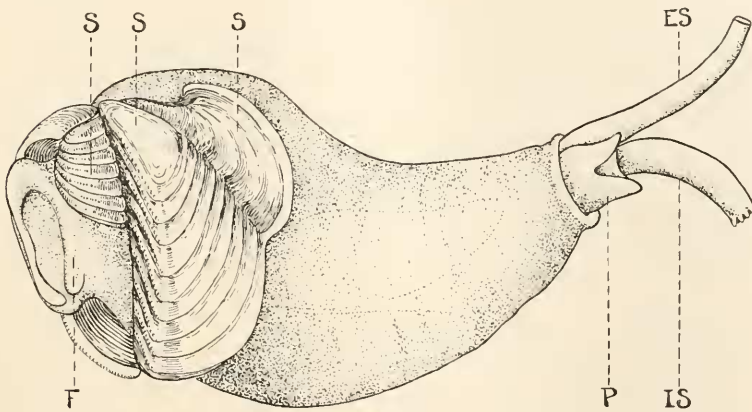


FIG. I. Young *Teredo*, length 2 cm., age five weeks from metamorphosis; drawn by camera lucida. S shell, F foot, i. s. incurrent siphon, e. s. excurrent siphon, p. pallet.

Effect of Adverse Conditions. (Repairing the burrow, etc.)

In case the tunnel is broken by accident, or by the wearing away of the surface of the wood from any cause, the adjacent glands secrete shell substance in greater abundance and mend the breach. The integrity of the burrow is carefully preserved. In case adverse conditions arise which make the environment difficult either from enemies or poisons in the water, or from overpopulation by its fellows, this shell substance is secreted in the form of a heavy casing, not only on the sides, but over the anterior burrowing end as well. This is the invariable reaction of

Teredo to adverse external conditions, the most common cause of which is the crowding of individuals in small timbers. As a consequence, the wood becomes extremely fragile, a mere shell, so porous that enemies, such as bacteria and parasitic protozoa, find entrance and menace the life of the community. Under these conditions the worms die within the first year. It may be, too, that wood is an essential part of their diet, but it is more probable that the trouble is a lack of adequate protection against adverse conditions and dangers from without.

No *Teredo* ever molests the burrow of another. When two come close together they face about and proceed in another direction, thus avoiding each other. When they become so closely crowded that further burrowing would infringe upon a neighbor, growth seems to stop. The size attained depends upon the amount of crowding. As stated above, the *Teredo* responds to these conditions by greatly thickening the shell lining of its burrow on the front as well as on the sides so that the whole is strongly encased. However, it is at best a brittle affair and parasitic protozoa and bacteria are admitted which soon destroy the occupant. The protozoan *Architophrya* (a holotrich) is always abundant in such situations.

It is difficult to see how growth may cease and the animal survive, but it is perfectly clear that *Teredo* three months old living in crowded situations are often less than one fifth as large as others of the same age growing under better conditions. The stunted worms, though packed closely together are frequently all alive and reproducing. As many as seven young *Teredo* per square inch have been observed in test blocks although the average is by no means so high. When these worms all become two or three inches long, a crowded group results unless they happened to have entered a large timber which permits of unlimited expansion.

Shipworms rarely go from one board to another, no matter how closely the boards are applied to each other. Only two exceptions to this rule have been observed among the thousands of burrows studied. They seem to avoid anything that threatens to interrupt the continuity of their tunnels.

Teredo seems not to orient to gravity since it burrows down-

ward about as frequently as upward. The burrow of a single individual often shows that there is no tropistic response of this kind. If in tunneling downward a *Teredo* approaches the end of the timber, another *Teredo* burrow or a knot, it may turn directly about and proceed in the opposite direction, paralleling the first part of its burrow. By some means it is able to detect any nearby surface of the wood and avoid it. Two *Teredo* tunnels may approach within an eighth of an inch of each other, but they remain quite separate. They have some sense also which warns them, when approaching the end of a timber, to face about before reaching the end, retreating usually at a point 5 to 10 mm. from the tip.

SECTION III. THE BREEDING SEASON.

My interest in *Teredo* dates from 1922 when the National Research Council suggested the study of the breeding season of this species and appropriated funds to meet preliminary expenses. The results of this study were reported at the Washington meeting of the American Association for the Advancement of Science in 1924, and an abstract was printed at that time. The publication of the paper as a whole was deferred until the study of various details could be completed.

The fact that the female carries the young embryos in the gill chamber for a short time makes an accurate study of the breeding habits a comparatively easy matter. It may be ascertained at a glance whether a female is carrying embryos or not and the presence of eggs or embryos in the suprabranchial chamber is conclusive evidence of recent spawning. A further useful indicator is that of color. The eggs and young embryos are pure white, but they gradually take on a dark gray color with age.

The first spawning at Woods Hole occurs from the first to the middle of May, and the last about the middle of October. During 1925 eggs were first obtained on May 15 and these were in a late cleavage stage when discovered. Two of twenty females examined had spawned at this date. In 1926 eggs were first obtained on May 16. Two of the twelve females examined had spawned, and the embryos were in the gastrula stage of development. Frequent previous examinations in April and May had shown no spawning individuals.

During the fall of 1925 and 1926 special trips were made to Woods Hole in order to determine the extreme limits of the breeding season. At this time an effort was also made to learn how late in the fall veligers were metamorphosing and entering wood. On September 22, 1926, numerous females, both in Eel Pond and at the Cayadetta Wharf in Vineyard Sound, were carrying embryos in various stages of development. On October 10, of sixty *Teredos* taken from Eel Pond, none were carrying embryos, while five of twenty five taken from the Sound had quantities of veligers in their gills. The embryos of one of these were late trochophores or early veligers and repeated observation on the rate of development in *Teredo* has shown that these would normally be carried from ten days to two weeks longer. None were found carrying embryos on November 4. These and other data show that the breeding season in Eel Pond ended two weeks earlier than in Vineyard Sound. The difference in temperature is apparently the cause of this diversity in the duration of the breeding season, Eel Pond being approximately two degrees colder during the fall than the deeper water of the Sound. Kofoid noted a similar difference in the breeding season in various parts of San Francisco Bay where wide stretches of shallow water become several degrees warmer in early spring and cooler in the fall than the deeper portions of the same body of water. His estimate of two weeks difference is no doubt conservative. Observations just completed at this writing show that the first spawning by *Teredo* in Eel Pond in 1927 occurred on May 1 and in Vineyard Sound on May 12. Spawning occurred in each case when the water had reached a temperature of approximately 11° C. (between 11° and 12° C.). Since spawning ceased in Eel Pond on October 1 and in Vineyard Sound about October 15 we have the same variation due to temperature difference and the total spawning season for *Teredo* at Woods Hole is shown to be nearly or quite five months in duration.

It should be explained that the larva has a free swimming period of approximately two weeks after leaving the supra-branchial chamber of the mother before it is ready to enter wood. In accordance with the fact that veligers are carried by the mother as late as October 20 in Vineyard Sound, one would expect to

find that wooden structures are being entered by the metamorphosing veligers until the first of November. The facts, however, do not bear out this expectation. The last date on which veligers successfully metamorphosed and attacked wood in Eel Pond was September 23, whereas larvæ were no doubt present until about October 5. Lobster pots placed in Vineyard Sound on October 10 were entered by metamorphosing veligers. It is certain that larvæ are present in the water in Vineyard Sound until November 1 or the last week in October. In other words, larvæ are present in the water at least two weeks after the last ones successfully attack wood. The reason for this is not evident. The cilia of the swimming mechanism of the larva possibly become less and less active as the water cools, with the result that mortality among the last generation of larvæ of the season is high. In *Bugula* also the last larvæ of the season fail to metamorphose, but not to so great an extent as is the case with *Teredo*.

An examination of the gills of a large number of *Teredo* on November 4 showed a spotting of these organs as if the last embryos contained had been resorbed. It is quite likely that the belated ones lose ability to swim and therefore remain inactive and disintegrate in the gill chamber. (This may not be the correct explanation of the cause of the failure of the last embryos of the season to metamorphose.) The larvæ of *Bugula* and those of certain hydroids continue to metamorphose successfully into November and the latter into December although dependent upon cilia for locomotion.

The data in hand indicate that the breeding season of *Teredo* at Woods Hole extends from about May 10 to October 10 or possibly to October 15, a period of five months.

Fecundity.

Teredo is tremendously prolific. Each female spawns three or four times in a season. The number of eggs produced varies with the size of the individual and is estimated to be from one to five millions. At the end of the season the female seems to be exhausted. Many molluscs survive for several years but *Teredo* dies during the second year as test blocks have shown repeatedly. This unusual fecundity may explain the early loss of vitality.

As evidence that the female *Teredo* spawns every four or five weeks, the following data are offered. Several cases of this kind were observed.

June 20, 1925. Two large females which were carrying gray veligers, were ready to spawn a second time. The ovaries were large and distended with eggs which were full size and fertilizable.

June 24, 1925. Two among several females examined had spawned a second time this season, numerous late veligers mixed with cleaving eggs were found in the suprabranchial chamber.

Periodicity.

One of the specific objects of this study was to ascertain the characteristics of the breeding season, whether or not there is a lunar or other periodicity in the production or shedding of the gametes. It was made apparent during the first year's study that no lunar periodicity occurs in the spawning of *Teredo*. From the beginning to the end of the breeding season, the water contains abundant larvæ in all stages of development. The records of examinations of hundreds of stakes and lobster pots indicate that larvæ are abundant in the water ready to attack any exposed timber each day of the summer. The evidence bearing on this point is derived from two types of experiments which are here described in some detail because other workers have stated that the spawning of *Teredo* is periodic and that definite broods mature at definite times.

1st. The following tables show that no periodicity in the spawning by this species occurs. Of a large number of ship worms that may be examined at any time during the summer, some will be found to carry cleaving eggs, some gastrulæ, and some trochophores, some young veligers and some typical veligers, thus showing that spawning is continuous and not synchronous.

TABLE I.

Teredo EXAMINATIONS 1925, JULY 1.

Material from Lobster Pot Placed in Water Aug. 16, 1924. Ecl Pond.

Females carrying unspawned eggs	4
Females carrying cleaving eggs in gill chamber	1
Females carrying young veligers in gill chamber	1
Females carrying typical veligers in gill chamber	4
Mature males with active sperm	7
<hr/>	
Total	17

TABLE 2.

Teredo EXAMINATIONS 1924, JULY 5.*Material from Lobster Pot Placed in Water Aug. 20, 1923. Cayadetta Dock.*

Females carrying mature eggs	5
Females carrying immature eggs	1
Females carrying cleaving eggs in gill chamber	7
Females carrying gastrulæ in gill chamber	5
Females carrying young veligers in gill chamber	4
Females carrying typical veligers in gill chamber	5
Mature males having motile sperm	7
Immature males	3
Total	37

TABLE 3.

Teredo EXAMINATIONS 1924, JULY 19.*Material from Lobster Pot Placed in Water Aug. 20, 1923. Cayadetta Dock.*

Females carrying eggs	8
Females carrying cleaving eggs in the gill chamber	2
Females carrying gastrulæ in the gill chamber	11
Females carrying young veligers in the gill chamber	3
Females carrying typical veligers in the gill chamber	5
Mature males with active sperm	5
Total	34

TABLE 4.

Teredo EXAMINATIONS 1924, AUG. 10.*Material from Lobster Pot Placed in Water Aug. 20, 1923. Ecl Pond.*

Females carrying eggs	1
Females carrying cleaving eggs in the gill chambers	1
Females carrying blastulæ or gastrulæ	4
Females carrying early veligers	2
Females carrying typical veligers	5
Females carrying a few veligers in the gill chambers, apparently spent	1
Mature males with abundant active sperm	12
Total	26

These four tables show that spawning takes place at all times during the month and not synchronously. They show conclusively that there is no lunar or other periodicity such as that sometimes caused by variations of temperature. Attention is

called to the fact that the spawning of these animals took place not in the laboratory, but normally in their natural habitat.

It is also apparent from these tables that there are no "broods" or special times of infestation of exposed timber. As further evidence on this point the test blocks (lobster pots) were put out every ten days during the summer and all became infested with metamorphosing *Teredo* larvæ almost at once, certainly within a day or two after exposure, as numerous experiments on rate of growth show. At Woods Hole the first larvæ settle and begin to burrow toward the end of June (June 20). From that time on until early fall the water contains a copious supply of swimming larvæ ready to burrow into any exposed wooden structure.

T. C. Nelson in his report for the year 1923, Table 5, page 208, concludes on very meager and insufficient data that one brood of larvæ settled in Barnegat Bay in June and that a second brood matured some time between July 26 and September 4. The evidence derived from my experiments covering four years show that there are no broods but rather a continuous entrance of timbers by larvæ maturing throughout the breeding season. The evidence of many experiments shows that one can not depend upon green timber or even seasoned 2×4 stakes for such experiments, as they may remain uninfested for weeks for no apparent reason. Seasoned lobster pots, however, regularly became infested either the day they were exposed to the water or very soon thereafter. This is possibly due to the horizontal position of the timbers in the water, as contrasted with stakes standing vertically. The answer to the question whether *Teredo* larvæ enter wooden structures in broods at special times or continuously has important practical bearings as well as scientific interest.

It is also apparent from the data of these tables that Nelson's statement that there are five hundred females to one male, does not hold for the Woods Hole region. Females outnumber males but by no means to so great an extent.

Kofoed has shown that the number of larvæ in any particular region depends upon the extent to which infested timber is present. Regions far from wooden warves have relatively few larvæ in the water. I was able to show that *Teredo* is much more abundant at the Cayadetta Wharf than in Eel Pond, the

ratio being approximately 2:1. The distance between these locations is less than one hundred yards and the difference in numbers in this case is not due to a difference in the amount of wood present. The biological conditions in the more or less isolated Eel Pond are clearly different from those of the open waters of Vineyard Sound because species inhabiting them are different to some extent, as shown in another paper (See *Bugula*).¹ A study of these conditions is contemplated but at the present no adequate explanation is suggested unless the large amounts of formalin and other poisons and oils from the supply station seriously affect the Eel Pond water at times. There are, however, differences in natural conditions. The tidal currents outside, at any rate, are much stronger than those in Eel Pond.

SECTION IV. EMBRYOLOGY, AND RATE OF DEVELOPMENT.

The extensive contributions of Sigerfoos and Hatschek give satisfactory descriptions of embryological development so that I shall avoid duplication and emphasize only facts that are new.

The egg of *Teredo* is comparatively small and white in color. It measures in extreme limits from .050 mm. to .061 mm. with an average diameter midway between these figures. The oviducts open into the suprabranchial chambers which are extensive and serve as brood pouches. When the eggs are extruded they are retained in the suprabranchial chambers for a period of two or three weeks, during which time they pass through the early stages of development. When liberated into the sea water they are typical lamellibranch veligers, vigorous and hardy. A large female may liberate from 500,000 to 1,000,000 eggs at a single spawning, so that the gill chambers are tightly packed with embryos distributed in two parallel rows along the sides of the slender elongated body. The approximate age of embryos can be estimated by their color since they gradually change from white to a dark muddy gray during development.

The embryo is not parasitic upon the mother, but the egg will not develop outside the gill chamber. Ripe eggs were several times removed from the gonads and artificially fertilized in an

¹ *Bugula flabellata* lives readily in Eel Pond but will not thrive in the adjacent waters of Vineyard Sound, while the reverse is the case with *B. turrita*.

attempt to observe them in development. Development was initiated but no egg cleaved beyond the sixteen cell stage, and many stopped at the two, four, and eight cell stages. Development in these cases was extremely slow and cleavage was irregular and abnormal. Eggs fertilized at six P.M. had reached the eight cell stage at 9 P.M. It is probable that development in this species is normally slow, but this rate can hardly be considered normal. Very young embryos in the two and four cell stages were several times found in the suprabranchial chambers and these when removed developed no better than the artificially fertilized eggs. In common with artificially fertilized eggs, they finally became viscid and adhered to the containing dish. It was found also that blastulæ and gastrulæ would fare no better. They failed to develop into swimming larvæ. Late trochophores and early veligers on the other hand continued to develop normally when removed from the gill chamber to sea water. Veligers removed prematurely showed great vigor and swimming ability, and were several times kept for two weeks in sea water. Veligers withstand much rough treatment and survive in poorly aerated and even foul water. Some were kept in glass aquaria and fed on diatoms for three weeks, but to what extent they metamorphosed and entered the wood that was provided was not learned.

The gastrula is invaginate, similar to that of many other molluscs and annelids that produce small eggs with little yolk. The trochophore is especially interesting because in adaptation to its parasitic mode of life, it fails to develop a strong protroch. The cells which normally develop this larval swimming organ are undoubtedly present and distributed in a broad equatorial band similar to that of many molluscs, and they are more extensive than in most annelids. The protroch is apparently present and was described by Hatscheck. I found it either absent or so feebly developed as to be easily overlooked. The trochophore is pear shaped or slightly elongated and on the average measures $.059 \times .060$ mm. in length. As it begins to transform into the veliger, strong cilia develop on the velum, and the embryo becomes motile long before it is ready to be expelled into the sea water to shift for itself.

Duration of Larval Period.

Sigerfoos failed to find free swimming veligers in the water and both he and Nelson speak of the habits and duration of the larva as being unknown. The larval period from fertilization to metamorphosis has usually been estimated at about one month. It is evident, however, that it varies somewhat with temperature being shorter in tropical and sub-tropical regions than at Woods Hole.

I have frequently found *Teredo* veligers, in various stages of development, settling upon horizontally placed boards and Nelson has more recently taken them in "tow," as well as hovering about piles ready to settle permanently. In fact, he corroborates the observations of Harrington that the mature veligers of *Teredo* are attracted to wood and wood extracts. The duration of the free swimming period has never been accurately determined. To give attention to this phase of the life history publication of this paper has been delayed until its study could be completed and verified. The evidence now at hand indicates that the entire developmental period from egg to metamorphosing larva, is about five weeks. At least half of this time is required for development in the gill of the mother, leaving for the free-swimming period not to exceed two or three weeks. The evidence on which this conclusion is based is derived in various ways but is indirect. Since the method and conclusion may be questioned, the data are explained in considerable detail in the following pages.

In 1925 the first eggs were laid between May 12 and May 15, while the first young metamorphosed *Teredo* were found in test blocks on July 2 and July 5. These young, metamorphosed shipworms measured .35 mm. to .5 mm. Evidence collected from many experiments carried out during the past two years shows that young *Teredo* of this size have spent from 15 to 18 days in the wood, or rather, that they settled and began to metamorphose and burrow 15 to 18 days previously. If we subtract 15 days from July 2 or 18 days from July 5, June 17 is the approximate date when these *Teredo* ended their careers as free swimming larvæ. Other young *Teredo* collected on July 7 measured 1 mm. and these are known to be three weeks old, or that three weeks

had elapsed since they settled upon wood. Subtracting twenty-one days from July 7 gives the date June 17 when metamorphosis began. The total larval period is therefore between four and five weeks. Other data collected during 1925 lead to the belief that the time is more nearly five weeks than four. This method, though indirect, is accurate, and was repeated many times at the opening of the breeding seasons of 1925 and 1926. The experiments made to determine the rate of the metamorphosis and rate of early growth were also repeated many times during the past two years and are also reliable. They show conclusively that young *Teredo* which measure one millimeter are approximately three weeks old. The spring of 1925 opened unusually warm, although the winter was severe, so that animals came out of winter hibernation a few days earlier than usual. The effect of this was shown most markedly in the rapid somatic growth of many animals, but it also affected to a slight extent the breeding seasons of most animals. The date of first settling of *Teredo* larvæ at Woods Hole is usually about June 20, and the first spawning about May 10. The variation in the spawning season from year to year does not usually exceed two or three days but it may vary more than a week. There is evidence that some animals begin to breed only when the water rises to a certain temperature. This, however, is by no means a universal rule.

Rate of Growth.

The veliger of *Teredo* has the typical form common to lamelli-branch larvæ, but is not so thick or nearly spherical as sometimes described. Young veligers taken from the gills in an early stage of development measure on the average .065 x .080 mm. Five specimens taken from two individuals measured as follows: .060 x .080 mm.; .065 x .080 mm.; .065 x .082 mm.; .070 x .083 mm.; .070 x .085 mm. These measurements represent the range of variation in length and breadth. One of these seen in edge view measured .082 x .05 mm., and an older one .09 x .05 mm. Veligers ready to begin their free swimming life, after spending two or three weeks in the gill of the mother, measure somewhat larger, as the following examples show: .070 x .090 mm.; .072 x .090 mm.; .075 x .085 mm.; .075 x .088. These measure-

ments not only indicate some growth but also that a considerable variation exists in the relative measurements. The range of variation in ratio of length to breadth and also in length of hinge line is great. Veligers fed upon diatoms for one week measured from .077 x .090 mm. to .081 x .093 mm. The size attained at the time of metamorphosis was not learned, but Nelson ('23) gives it as .25 mm. in length. I have collected several hundred young metamorphosing *Teredo*, which had burrowed into wood, varying in age from two to three weeks after settling. These range in size from .35 mm. to 1 mm. The smaller ones in two weeks have almost completed metamorphosis and have from two to three rasping ridges or rings of growth on the shell. Individuals three weeks old have four rings of growth and a typical *Teredo* shell. At three weeks of age the *Teredo* is practically spherical and its burrow, when exposed by cutting away the surface of the wood, is a hemispherical pit. The young worm now begins to elongate rapidly and at the end of one month its burrow measures from 5 to 7 mm. in length, and has a diameter of 2 to 2.5 mm. The shipworm when expanded fills its burrow so that, in measuring the rate of growth, the size of the burrow may be taken as the correct measure of the enclosed worm. When the shipworm is removed from its burrow, it contracts to one half or two thirds of its expanded measure. Tables 5 and 6 show the rate of growth from the egg to adult size, and need not be described in detail. Measurements were taken every three or four days, and the rate of increase in size from day to day was found to be surprisingly rapid.

It should be noted that the ages given in the tables include only the time that elapsed from the time of settling. If the age from the egg is desired, about thirty-five days should be added to these figures to include the time from fertilization to the end of the free swimming period. The larval period is excluded in the following description and from the tables.

Growth during the first twenty-five days seems small but when the minute size of the animal at the beginning is taken into account, the growth is not slow. From one month to five months the increases shown during the intervals of three or four days, between measurements, are seen to be remarkably great. For



TABLE 5.

RATE OF GROWTH OF *Teredo navalis* (SUMMER).

Measurements of the Largest Burrows of Specified Ages.

Date and Period of Growth.	Age.	Size of Burrows in Length and Widest Diameter (Metric).	Approximate Length in Inches.
July 16 to July 26....	10 days	No visible <i>Teredo</i> burrows.	
July 16 to Aug. 3....	18 "	No visible <i>Teredo</i> burrows.	
July 26 to Aug. 13....	18 "	.35 to .5 mm. x .35 to .5 mm.	.014 to .02 in.
July 16 to Aug. 8....	23 "	.35 to .5 mm. x .35 to .5 mm.	.014 to .02 in.
July 16 to Aug. 11....	25 "	.5 to .1 mm. x .5 to 1 mm.	.02 to .04 in.
July 26 to Aug. 20....	25 "	1 to 1.5 mm. x 1 to 1.5 mm.	.04 to .06 in.
July 16 to Aug. 13....	28 "	2 to 3 mm. x 1 to 2 mm.	.08 to .12 in.
July 16 to Aug. 16....	30 "	5 to 7 mm. x 2 to 2.5 mm.	.2 to .28 in.
July 16 to Aug. 18....	33 "	8 to 10 mm. x 2 to 3 mm.	.32 to .4 in.
July 16 to Aug. 23....	38 "	14 to 17 mm. x 3 mm.	.56 to .7 in.
July 16 to Aug. 28....	43 "	35 to 45 mm. x 4 mm.	1.4 to 1.8 in.
July 16 to Sept. 1....	46 "	50 to 57 mm. x 4 to 4.5 mm.	2 to 2.3 in.
June 20 to Aug. 20....	60 "	70 to 75 mm. x 4 to 4.5 mm.	2.8 to 3 in.
July 3 to Sept. 6....	65 "	80 to 90 mm. x 4 to 4.5 mm.	3.2 to 3.6 in.
June 22 to Sept. 3....	72 "	100 to 120 mm. x 4.4 to 4.8 mm.	4 to 4.8 in.
June 22 to Oct. 20....	130 "	140 to 170 mm. x 6.5 to 7 mm.	5.6 to 6.8 in.
June 22 to Dec. 1....	160 "	175 to 200 mm. x 7 to 7.5 mm.	7 to 8 in.
July 1923 to July 1924	One year	250 to 400 mm. x 7.8 to 9.4 mm.	10 to 16 in. x $\frac{5}{16}$ to $\frac{3}{8}$ in.

Note.—The left hand columns of Tables 5 and 6 represent the time of exposure of timber to the sea water. Thus, if we consider the first item of Table 5, timbers were exposed to sea water on July 16 and examined for *Teredo* on July 26, making ten days as the maximum age of the infesting *Teredo* as indicated in the second column of the table. Column three gives the measurements of the infesting *Teredo* if any.

The measurements given in these tables are actual cases and not averages. Many more were measured than are given here but the data given are considered typical.

In all cases the larval period is omitted. The age from fertilization may be approximated by adding thirty-five days to the age as given here.

Timbers exposed forty-three days, July 16 to August 28 contained sexually mature worms. Other similar data show that *Teredo* under favorable conditions becomes sexually mature in six weeks at Woods Hole. The item second from the bottom of Table 5 shows that *Teredo* does not reach adult size during the first season (June 23 to December 1). No growth takes place after December 1. (See also Table 6.) The last item of the table shows that adult size is attained in one year (July 1923 to July 1924). The largest specimen found at Woods Hole measured forty centimeters in length. It was precisely one year old.

example, the length of the largest burrows at twenty-five days is 1.5 mm., at thirty days 5 to 7 mm., at thirty-eight days 14 to 17 mm., at forty-three days 35 to 45 mm. Davenport claims that

growth in size is partly due to swelling by the absorption of water. It is easier to account for this phenomenal growth in this way than to suppose that the change in size represents only protoplasmic growth and actual cell multiplication.

It has been repeatedly found that *Teredo navalis* at Woods Hole reaches sexual maturity and spawns from six to eight weeks after entering the wood as a metamorphosing larva. The youngest to spawn were six weeks old, and their burrow measured one and one half inches (38 mm.) in length. They spawned in abundance at the age of two months when they measured 2-2½ inches (50 to 63 mm.) in length. Sexual maturity is reached long before adult size is attained, since a fully developed shipworm measures from 12 to 16 inches (30 to 40 cm.) in length.

The larvæ that metamorphose first in a season almost reach adult size by December 1, the largest ones, measuring from seven to nine inches (17.5 to 22.5 cm.) in length. During December, January, February and early March ship worms are practically dormant and do not grow perceptibly. Then they may be said to awaken and by the first of July the oldest have reached maximum size. The largest specimen found at Woods Hole measured 16 inches in length and ¾ inch in widest diameter (40 x 1 cm.). It grew in a 2 x 4 test take which was in the water from July 1, 1923 to July 1, 1924. Others measuring from 12-15 inches are common. Larvæ which enter the wood later in the summer, even to October 1, lie dormant over winter and resume growth the following spring. It was shown that growth is greatly retarded after the first of November, especially on the part of the youngest *Teredo*. Table 6 shows the rate of growth for the entire year including the winter. It has already been stated that *Teredo* reaches adult size in one year and dies during the second year.

Teredo navalis is said to grow to a slightly larger size in sub-tropical climates than at Woods Hole. As stated above the largest specimen found in this northern locality in four years' study measured forty centimeters in length and one centimeter in greatest diameter.

TABLE 6.
RATE OF GROWTH OF *Teredo navalis* (EFFECT OF WINTER UPON GROWTH).
Measurements of the Largest Burrows of Specified Ages.

Period of Growth or Time of Exposure of Timber.	Age.	Size of Burrows in Length and Widest Diameter (Metric).	Length and Width of Burrows in Inches.
Nov. 1 to Dec. 1.....	1 month	No teredo infestation.	$1\frac{1}{4}$ to $1\frac{3}{4}$ in. x $\frac{5}{32}$ to $\frac{3}{16}$ in.
Oct. 15 to Dec. 1.....	$1\frac{1}{2}$ "	No teredo infestation.	3 to $3\frac{1}{4}$ in. x $\frac{1}{16}$ to $\frac{7}{32}$ in.
Oct. 1 to Dec. 1.....	2 "	No teredo visible.	4 to $4\frac{1}{16}$ in. x $\frac{1}{4}$ to $\frac{3}{32}$ in.
Sept. 15 to Dec. 1.....	$2\frac{1}{2}$ "	No teredo visible.	$4\frac{1}{2}$ to $5\frac{3}{4}$ in. x $\frac{1}{4}$ to $\frac{9}{32}$ in.
Sept. 1 to Dec. 1.....	3 "	No teredo visible.	6 to $8\frac{1}{2}$ in. x $\frac{1}{4}$ to $\frac{3}{32}$ in.
Aug. 20 to Dec. 1.....	$3\frac{1}{2}$ "	35 to 45 mm. x 4 to 4.5 mm.	7 to 10 in. x $\frac{1}{4}$ to $\frac{3}{32}$ in.
Aug. 10 to Dec. 1.....	$3\frac{3}{4}$ "	75 to 81 mm. x 4.5 to 5 mm.	10 to 16 in. x $\frac{1}{16}$ to $\frac{5}{8}$ in.
Aug. 1 to Dec. 1.....	4 "	100 to 110 mm. x 6.2 to 7 mm.	$1\frac{1}{16}$ to $2\frac{5}{8}$ in. x $\frac{1}{8}$ to $\frac{1}{6}$ in.
July 20 to Dec. 1.....	$4\frac{1}{4}$ "	112 to 144 mm. x 6.2 to 7 mm.	$\frac{1}{2}$ to $\frac{5}{8}$ in. x $\frac{1}{16}$ to $\frac{3}{8}$ in.
July 10 to Dec. 1.....	$4\frac{3}{4}$ "	159 to 210 mm. x 6.2 to 7 mm.	
July 1 to Dec. 1.....	5 "	175 to 250 mm. x 6.2 to 7 mm.	
July 1 to Dec. 1.....	12 "	250 to 400 mm. x 7.8 to 9.5 mm.	
Sept. 12 to July 1.....	Winter +	48 to 70 mm. x 3.3 to 4.1 mm.	
Sept. 23 to July 1.....	Winter +	12 to 31 mm. x 2.5 to 3.3 mm.	

Note.—Table 6 shows, primarily, the amount of growth attained by *Teredo* during the first season up to the beginning of the hibernation period (Dec. 1). It shows not only that the earliest larvæ of the season fail to reach maturity (adult size) the first season, but also gives the amount of growth attained by the later larvæ of the season as well.

The first five items of this table show that *Teredo* larvæ which enter the wood after September 1 will not attain sufficient size to be detected in the wood up to December 1. Timbers exposed to the water as late as August 20 on the other hand are likely to contain *Teredo* measuring 35 to 45 mm. in length by the end of the growing season (Dec. 1).

The last two items of the table show that timbers exposed to the water between Sept. 12 and Sept. 23 become infested by *Teredo* and that they appear in the wood the following July, although they remain too small during the winter to be detected.

The latest infestation observed at Woods Hole occurring in Eel Pond was Sept. 23, and in Vineyard Sound Oct. 10. It may sometimes occur somewhat later than this, since the point was not sufficiently investigated. Table 6 shows that growth in the late fall is very slow compared with summer growth as given in Table 5 and data not tabulated show that practically no growth takes place in *Teredo* at Woods Hole between Dec. 1 and March 1. Some observations indicate that the gonads begin to proliferate extensively before there is detectable body growth in the spring.

SUMMARY.

Teredo navalis occurs in abundance at Woods Hole and vicinity and has been known there for many years.

The breeding season extends from about May 10 to October 10. Spawning begins in the spring when the water reaches a temperature between 11° and 12° C. Spawning by each female occurs several times during the season. No lunar periodicity in spawning occurs and there are no broods caused by synchronous spawning.

The eggs are retained in the gills of the mother during cleavage and early larval development.

The time required for the fertilized egg to complete larval development to metamorphosis is approximately five weeks at Woods Hole. About half of this time is passed in the brood pouch and half as a free swimming veliger.

When eggs and early embryos are removed from the gills they do not develop normally.

The trochophore of *Teredo* is non-motile, having either a feebly developed protroch or none.

Teredo navalis reaches sexual maturity in six weeks or two

months after metamorphosis when it measures four or five centimeters in length. It reaches adult size in on year, and dies during the second year. The largest specimen collected in four years measured forty centimeters in length and one centimeter in greatest diameter.

The rate of growth during the summer months and also during the winter was determined and tabulated. Certain habits of shipworms were also observed and recorded.

APPENDIX.

Practical Measures.

Because of numerous inquiries by lobstermen and owners of small boats concerning methods of preventing damage by shipworms, a series of experiments was made on the effect of drying upon *Teredo*. These experiments were not extensive but sufficient to show that simple but effective precautions may be taken.

Teredo larvæ first begin to enter wood between June 20 and June 25 and stop about October 10. Shipworms do not enter wood at any other time during the year. Little or no damage is done to wood until it has been in the water one full month. The largest of the young shipworms are only one fourth of an inch long at the end of one month but they attain a length of one inch in six weeks. It is, therefore, advisable to dry lobster-pots and boats once per month and leave them out of the water exposed to the sun for one week. This is especially true in July and August when most of the damage is done. Shipworms in small timbers are killed by five days' exposure to sunlight, but 2 x 4 stakes and larger timbers require from a week to ten days for drying sufficiently to kill all of the worms.

EXPERIMENTS.

(1) Infested lobster-pot lath:

- (a) After exposure in air to bright sunlight for 1 day, seemed to be dry but some of the infesting shipworms were still alive.
- (b) After exposure in air to bright sunlight for 2 days, some shipworms alive.

- (c) After exposure in bright sunlight for 3 days, all shipworms dead.
- (2) Infested lobster-pot lath:
- (a) After 7 days on shelf in laboratory, all worms contracted and shrunk, some of which regained plumpness and normal activity when placed in sea water. Spermatozoa and larvæ taken from these shrunk worms showed activity.
- (b) After 10 days on shelf in laboratory—all worms, sperm and larvæ dead.
- (3) Infested 2 x 4 stakes:
- (a) After exposure in air to sunlight for 5 days; many shipworms dead, some living.
- (b) After exposure in air to sunlight for 7 days; all shipworms dead.
- (4) Infested 2 x 4 stakes:
- (a) After exposure in air in shade for 7 days; many worms dead but some living.
- (b) After exposure in air in shade for 10 days; none living.

Note.—If infested 2 x 4 stakes are exposed in air in the shade but kept wet some worms may live for several weeks.

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STUDIES OF HUMAN TWINS.

I. METHODS OF DIAGNOSING MONOZYGOTIC AND DIZYGOTIC TWINS.

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INTRODUCTION.

This is the first of a series of studies of human twins based upon a considerable collection of pairs taken from the environs of Chicago. These studies have been carried on in collaboration with Professors F. N. Freeman, K. J. Holzinger, and Mrs. Blythe Mitchell. The original objective of this research project was to secure an adequate collection of monozygotic and same-sexed dizygotic twins about whose diagnosis we could be certain. With this objective attained, it was proposed to make an intensive comparative psychological study of the two types of twins to determine, if possible, the influence of heredity and environment upon the various mental traits. This is an old and somewhat hackneyed problem, but one that has never been at all satisfactorily solved. It seemed to us, however, that all previous studies had been inadequate because methods of diagnosing the two types of twins were unsatisfactory. The one crying need then was for a satisfactory method of diagnosing monozygotic twins, and the working out of such a method was assigned to the present writer.

COLLECTION AND CLASSIFICATION OF MATERIAL.

The objective set by the collaborators in this study was the collection of fifty pairs of identical twins and fifty pairs of fraternal twins. In order to simplify our task, we decided to eliminate the disturbing factor of sex dimorphism, and therefore confined our study to twins of the same sex, pairs in which the twins were both boys or both girls.

At first no selection was practised among same-sexed twins,

but all cases were taken as they came. As each case was completed an informal vote of the three or four workers present was taken as to the category (identical or fraternal) to which the pair belonged. Rarely, if ever, was there any difference of opinion, but in about one tenth of the cases there was some uncertainty and these cases had to be studied more intensively.

It soon appeared that the collection of identicals and fraternal was not going evenly, the fraternal being more numerous. If our preliminary judgments as to their classification were accurate we would need to stop the collection of fraternal and collect only identicals during the last stages of the period of study.

When the adjudged "fraternal" mounted to fifty-two cases (consisting of twenty-four male pairs and twenty-eight female pairs) there were only forty-three "identical" (consisting of twenty-five male pairs and eighteen female pairs). The sex ratio at that time was very close to normal expectancy: forty-nine male pairs to forty-six female pairs. The question arose as to whether the proportion of identicals to fraternal was running according to theoretical expectancy.

Various methods have been used to determine the proportion of monozygotic to dizygotic twins. One method involved the examination of the fetal membranes of considerable numbers of twin births in institutions where competent observers were able to secure these important diagnostic data. Spät in 1860 reported that, in a total of one hundred eighty-four cases of twins examined as to the membranes, 24.6 per cent. were monozygotic. Brem in 1891 reported 22.7 per cent. of monozygotic twins out of one hundred twenty-six twin births. Kralin in 1891 reports 19 per cent. of monozygotic twins among one hundred twenty-seven twin births, but includes as monozygotic two opposite-sexed pairs. Tigges found in 1896, 21 per cent. of monozygotic twins among fifty-two twin births, and Quenzel in 1894 reported 20.4 per cent. of monozygotics among one hundred eighty-one pairs of twins. These percentages range from 19 per cent. to 24.6 per cent.

A second method used by several investigators for computing the proportion of identical twins is statistical in character. The best known of these methods is Weinberg's "differential method."

In 1902 Weinberg described his method as follows: "Assuming that sex is determined at the time of fertilization and that about half of all zygotes will produce males and the other half females, it follows that there will be equal numbers of same-sexed as opposite-sexed fraternal twins. If, therefore, we double the number of opposite-sexed twins and subtract the product from the total of all twins, the remainder will represent the number of monozygotic twins."

Applying this method to large masses of twin data he found that the percentage of monozygotic twins varies from 23.4 per cent. to 31 per cent., the percentage differing in different countries. This agrees rather closely with the percentages determined on the basis of fetal membranes.

Recently Knibbs (1926) has worked out a formula for computing the number of monozygotic twins in the twin population, using data taken from the census of Germany. His formula is as follows: The ratio of monozygotic twins to all twins is $(M + F - P) \div (M + F + P)$, where M is the number of ♂♂ pairs, F the number of ♀♀ pairs, and P the number of ♂♀ pairs. This method gives 24.4 per cent. of monozygotic twins in Germany from 1906 to 1911.

Applying Knibbs' method to the extensive twin data for the United States that is presented by Nichols (234,497 ♂♂; 264,098 ♂♀; 219,312 ♀♀), we discover that 26.42 per cent. of this large group are monozygotic and that nearly 42 per cent. of all same-sexed twins are monozygotic.

The question now arises as to whether our small random collection of ninety-five pairs of same-sexed twins was composed of the expected number of identical and fraternal pairs. According to our diagnosis there were forty-three pairs of identicals and fifty-two pairs of fraternal—*i.e.*, 45 per cent. identicals instead of the expected 42 per cent. This is but a small discrepancy and may have two meanings: Either the random selection of twins has brought in two or three too many pairs of identicals or else some two or three of the pairs diagnosed as "identicals" should be classed as "fraternals." It is probable that the former explanation is correct, for it is very unlikely that the ideal ratio as determined on the basis of 717,907 pairs of twins would be

realized exactly in the first ninety-five cases selected at random. In fact, the close approach to theoretical expectation actually realized is almost too close. The conclusion may then be drawn from this that our methods of diagnosing identical and fraternal pairs cannot be far astray.

In order to complete the proposed collection of fifty pairs of identicals and fifty pairs of fraternal, it was then necessary to select seven cases of certain identical twins. Two cases of fraternal were eliminated from the fifty-two cases of fraternal in order to get down to fifty cases. The two cases eliminated were chosen for the following reasons: In one case one twin had lost three fingers and his palm was so scarred that no adequate palm print could be taken; in the other case one of the twins showed up with an infected hand and no palm print could be obtained. Since, in our diagnosis of monozygosity, the palm prints were used as highly important criteria, it seems well to eliminate these two pairs in which the palm print evidence was incomplete. The two pairs eliminated were unequivocal cases of unlike fraternal twins.

We have now complete data on one hundred pairs of same-sexed twins, fifty of which have been classed as identicals and fifty as fraternal. No doubt some of our readers are wondering how we can speak so confidently about our ability to classify all of our cases as either identicals or fraternal. It may be said that the method was slow in taking shape and was arrived at only after intensive study of the materials.

DIAGNOSIS OF MONOZYGOTIC TWINS.

The majority of workers on human twins seem to have despaired of arriving at an adequate classification of twins into clean-cut categories: monozygotic and dizygotic. Years ago Thorndike found so much difficulty with his cases that he came to the conclusion that all twins belong to a single series and have a similar origin. Lauterbach, 1925, after the study of nearly two hundred pairs of twins, found himself unable to separate the same-sexed pairs with any assurance. He tentatively classified 59 per cent. of the same-sexed twins as monozygotic, a percentage much too

high, suggesting that he has included a good many cases of similar fraternal twins in his "identical" group.

The most recent study of twins is that of A. H. Wingfield (1928) who studied one hundred two pairs of twins selected at random from the public schools of Toronto and Hamilton, Ontario. Taking all pairs of twins as they came there were accumulated seventy-six like-sexed pairs and twenty-six unlike-sexed pairs. The expectation would be about 65 per cent. of like-sexed twins instead of about 74 per cent., the number found in this collection. It seems probable, therefore, that some unlike-sexed twins were overlooked. Wingfield made an attempt to separate the seventy-six like-sexed pairs into two groups, identicals and fraternal. His method was somewhat precarious. He classed as "identical" all those which seemed to himself and the teacher to have a higher degree of physical identity than siblings are likely to exhibit. "Only those pairs of twins showing practically indistinguishable physical traits, as judged by the teachers in the school and myself, were included in the identical group. While it is not absolutely certain that all pairs included in the identical group had identical heredity, the chances in favor of this being the case are very great." The fact that he classed as identical over 44 per cent. of all the twins in his group is surprising in view of the fact that the statistical expectation is only about 26 per cent. It seems probable then that Wingfield has included among the "identicals" several cases of similar fraternal twins. This is further suggested by the fact that he found a coefficient of correlation of only about $+0.90$ for this group as compared with $+0.95$ obtained for our identicals.

That it is possible to develop a method of distinguishing between identical and fraternal twins is strongly suggested by the fact that two European twin specialists claim to be able to make such a distinction with a high degree of infallibility.

Dahlberg (1926), in his monograph on "Twin Births and Twins from a Hereditary Point of View," makes this statement: "The following demands should be satisfied for a diagnosis of monozygotism for a grown-up pair of twins:

"1. That the appearance of the twins give an impression of very great resemblance or identity.

"2. That during childhood, neighbors, school-fellows, etc., have had difficulties in distinguishing them and have sometimes confused them.

"3. That the configuration of the ears does not show great dissimilarity.

"4. That the finger prints show a certain high degree of similarity.

"5. That the anthropological measurements do not show too considerable differences."

Siemen's method (1927) is somewhat more detailed and exacting. He takes the very sensible view that no single criterion of monozygotic origin is reliable, but that judgment in doubtful cases should be based upon identity in as many traits as possible. He emphasizes the rarity of really questionable cases. Many years of experience in the study of twins has developed in him such a degree of confidence in his method of diagnosis that he considers that he has been able to reach "a certain diagnosis in virtually every case of twinning."

He finds, as others have found before and since, that the great majority of all twins are either so completely alike or so markedly different that there is no question about their diagnosis. A careful study of the certain cases should furnish criteria for diagnosing the few doubtful cases. Thus a study of over a hundred pairs of unquestionable identical twins has resulted in the following "scheme" for diagnosing monozygosity:

- A. Traits in which one-egg twins practically always agree and in which two-egg twins agree only very rarely:
 - 1. Hair color and form.
 - 2. Eye color.
 - 3. Skin color.
 - 4. Downy hair of the body.
- B Traits in which one-egg twins differ only within narrow limits and in which two-egg twins usually differ more widely.
 - 5. Freckles.
 - 6. Appearance of blood in the skin.
 - 7. Follicular processes.
 - 8. Tongue (furrowed or not) and teeth.

C. Traits in which one-egg twins usually, and two-egg twins rarely show strong resemblance:

9. Form of face.
10. Form of ears.
11. Form of hands.
12. Body build.
13. Mentality.
14. Illness and abnormality.
15. Traits studied by special methods—finger prints, etc.

Our own method of diagnosis has been considerably influenced by the methods of Dahlberg and of Siemens, especially by the latter, but is somewhat different from any previously used. Our effort has been to combine the best features of all known methods.

After our own method was developed and while reading Wingfield's monograph, the writer noted a reference to a short note in *Science* by Taku Komai (1927) entitled "A Criterion for Distinguishing Identical Twins from Fraternal Twins." The criterion described has to do with finger prints and palm and sole prints of twins. "Generally speaking," he says, "the same hands or feet of the identical twins resemble each other more closely in their patterns than the two hands or feet of the same individual." This I have found to be very frequently true, but the formula needs modification, as will be shown below.

OUR OWN METHOD OF DIAGNOSIS.

The method of identifying monozygotic twins used in the present work may now be described in detail. A great deal of attention has been given to this matter, for we realize that the soundness of our conclusions as to heredity and environment depend upon the correctness of this diagnosis.

At the beginning, it may be said that in over 90 per cent. of our cases there was at no time any doubt as to their classification. The great majority of one type of twins are so strikingly similar that their monozygotic origin is obvious. Their resemblance is not confined to gross physical correspondence, but extends to tones of voice, gestures, and peculiar mannerisms. One soon becomes sensitized to the intangible correspondences of

identical twins and diagnoses them almost at a glance. The great majority of the other type of twins strike one at once as entirely unlike, often being more different than average brothers or sisters. About these there is no question after the first glance. Our ability to diagnose cases improved during the course of our study and we found that there was no difficulty at all in diagnosing the last half of the pairs that presented themselves. Two of the very early pairs were diagnosed doubtfully that, when reexamined after a year of experience, offered no difficulty at all. Two other cases were left uncertain because we allowed ourselves to be influenced by statements of the mother. About these cases there should never have been any question had the mother not been loquacious.

Out of one hundred two pairs of twins there was justifiable doubt about only six cases. These cases have all been diagnosed satisfactorily with the possible exception of No. 61, which still remains slightly uncertain.

The following are our criteria for diagnosing identical (monozygotic) twins.

1. They must be strikingly similar in general appearance including various intangible resemblances.
2. They must be essentially identical in hair color, texture and form.
3. They must have the same shade of eye color and form of iris.
4. They must have the same skin color and texture (complexion) except when one is more tanned than the other.
5. They must have no marked differences in features; shape of ears; shape, size and arrangement of teeth.
6. They must have hands of the same type and nearly equal in size.
7. The general microscopic character of the papillary ridges in fingers and palms must be essentially the same.
8. There must be stronger cross resemblance than internal resemblance in one or more of the details of finger and palm patterns.
9. The presence of reversed asymmetry in handedness or hair whorl in one twin is confirmatory evidence of monozygosity, but

its occasional presence in unlike twins is not to be taken as an indication of monozygosity.

A great deal of stress has been laid upon the diagnostic value of the palm and finger patterns. While this criterion alone is inadequate for certain diagnosis, it is surprising how few mistakes were made in our effort to diagnose monozygosity on this basis alone. In the first forty-two cases in which a judgment was attempted on the basis of palm and finger prints alone, there was disagreement in only two cases with the judgment based on general resemblance. Our method has been to classify all cases on the basis of the first six criteria and then to check this classification by criteria 7 and 8.

PALM AND FINGER PRINTS AS CRITERIA.

The intensive study of palm and finger patterns is perhaps the best single diagnostic aid. After a scrutiny of the first thirty or forty sets of palm prints the writer began to notice an important fact about the palm and finger patterns of strikingly identical twins: namely, that, instead of showing mirror-imaging of patterns (involving the resemblance of the right hand of one to the left hand of the other) the two hands of one of the twins were direct duplicates in major features of the two hands of the other. Specifically, the right hand of one twin is more like the right hand of the other than like own left hand, and the left hand of one twin is more like left hand of other than like own right hand. Thus cross resemblance between the two twin individuals is stronger than resemblance between the two hands of the same individual.

Among twins that are somewhat less alike the same rule holds in a somewhat modified form. Thus right hand of one twin may be like right of the other, or left of one like left of the other, but the close resemblance does not extend to both sides. In still other pairs of twins in which one is distinctly left-handed, there is a reversal of asymmetry, so that the right hand of each twin is like the left hand of the other. *In every pair of obviously monozygotic twins the rule holds that there is stronger cross resemblance between the hands of one twin and those of the other than between the two hands of the same individual.* The same

is true of ears, teeth, and other structures that show more or less asymmetry, but there is more detail in palm and finger prints and a more objective method of comparing them. In the case of the fingers the types of patterns have been formulated in all cases in order to obtain a qualitative basis of comparison, and the friction ridges in all patterns (following the method of Bonnavie, somewhat modified) were counted under binocular so that a quantitative comparison between the fingers of one hand and those of another is possible. In both qualitative and quantitative respects the rule that cross resemblance is stronger than internal resemblance holds, for identical twins.

The studies of palm main line formulæ and of the occurrence and varied expression of the six fundamental primitive patterns have been greatly facilitated by the study of a paper now in manuscript, the work of a considerable group of experts, entitled "A Study of Error in the Interpretation and Formulation of Palmar Dermatoglyphies," by Cummings, Keith, Midlo, Montgomery, H. H. Wilder and I. W. Wilder. Professor Cummings, evidently the guiding spirit of the group in this collaborative inquiry, has very kindly furnished me with a copy of the manuscript and has thus made it possible for me to study the palms of our twins with far greater efficiency than would have been possible without this assistance.

With few exceptions the same rules of cross resemblance apply to the palmar main lines and patterns that apply to finger prints. Most frequently the cross resemblance runs similarly in all four respects: in qualitative characters of finger patterns, in quantitative values of finger patterns, in palmar main line formulæ, and in the occurrence of palmar patterns. Sometimes the cross resemblance is obvious in only three of four respects, sometimes in two, or only one; but if it is greater between one hand of one twin and either the same or opposite hand of the other twin than in own hands, the rule is considered to hold good.

While it is of importance that the detailed analysis of the finger and palm characters of this collection of twins should be published, this is hardly the appropriate place for it. One or two separate papers devoted to a special presentation and analysis of these data are planned for subsequent publication.

At this time we must ask the indulgent reader to accept tentatively our criteria for diagnosing twins. With the publication of the complete data used in this diagnosis the methods used may be put to any test that seems necessary.

Applying the criteria of diagnosis above described to the six pairs of twins about which there was some doubt, three of them fell readily into the category of identicals and three were classified as similar fraternal. At the present time the writer feels quite confident as to the correctness of diagnosis of the whole collection. The cases that might be questioned by some are the three cases of similar fraternal just referred to. Before discussing the problems arising out of a study of identical twins, it seems advisable to devote a few paragraphs to the fraternal twins, especially to the three cases most difficult to diagnose.

THE DIAGNOSIS OF FRATERNAL TWINS.

Of the fifty-two pairs of fraternal twins in our collection, three may be classed as "similar fraternal," and twenty as "slightly similar fraternal," and twenty-nine as "unlike fraternal." None of the pairs show as much resemblance as the least similar of the identical twins. The only cases that could possibly be at all in question as to their classification are the three "similar" pairs, numbered 61, 15, and 74. Let us carefully scrutinize these rather crucial cases as to the possibility that they might be monozygotic twins of the less nearly identical sort.

Pair 61.—These girls at first impressed us with their similarity. They were dressed exactly alike, arranged their hair alike and had very similar coloring. In height there was but three eighths of an inch difference; there were two and three fourths pounds difference in weight. Head length of A was 13.95 mm., of B 14.35 mm.; head width of A was 17.7 mm., that of B was 17.9 mm. The hair of both was in general rather similar, but that of B was a shade darker, softer, finer and not so heavy. Eye color was the same in both, a type of hazel. There was no difference in skin color. Ears of A were higher and narrower than those of B, and had a shorter lower lobe. A has fuller lips; B has the longer, more prominent chin. A holds eyes wide open; B has them nearly half closed. Bridge of A's nose more bowed

than that of B. The teeth of the two differ rather sharply, the upper arch of B being narrower and the teeth crowded and irregular, while those of A are regular.

The finger print formulæ are decidedly different :

<i>Left Hands.</i>	<i>Right Hands.</i>
1, 2, 3, 4, 5	1, 2, 3, 4, 5
A—U, R, A, W, W	A—U, U, U, U, U
B—W, U, R, U, U	B—W, R, U, U, U

The quantitative values of the finger prints are :

A—right hand 24	A—left hand 28
B—right hand 38	B—left hand 25

All four palm main line formulæ are different and the patterns are also different.

<i>Left Hands.</i>	<i>Right Hands.</i>
A—(9.8.5".5') B.O.O.O.O.	A—(11.9.7 .5') B.O.O.L.O.
B—(9.8.5".3) A.O.O.O.O.	B—(9.7.5".3) C.O.O.O.O.

Both are equally right-handed and both have clockwise hair-whorl.

In spite of a superficial rather close resemblance, then, there is no indication that these twins have had a monozygotic origin. This was the most difficult case to diagnose, but there seems now no doubt that these twins are dizygotic in origin.

Pair 65.—This case was somewhat puzzling because the two girls are both rather peculiar in appearance and are similar in many peculiarities.

In height A is 57½ inches, B 56⅞ inches. In weight, A is 113¼ pounds, B is 111¼ pounds. Head length of A is 14.5 mm.; that of B is 14.4 mm.; head width of A is 17.7 mm.; that of B is 17.1 mm. Hair of both is the same in color and texture; eye color of both is of the same shade of blue; B has a lower brow and a sullen expression about the eyes, while A has a contented expression. The skin is somewhat more florid in B. The ears of the two differ greatly, B having much longer lower lobe. The hands differ in shape, those of A being broader and thicker. B has shorter, more turned-up nose, a distinctly wider mouth, fuller lips and fatter face. The teeth differ radically, the upper arch of B being wider and straighter across the front and with wider teeth.

The finger print formulæ read as follows :

<i>Left Hands.</i>	<i>Right Hands.</i>
1, 2, 3, 4, 5	1, 2, 3, 4, 5
A—W, R, W, W, U	A—W, W, W, W, U
B—W, W, U, W, U	B—W, W, W, W, U

The quantitative values of the finger prints are:

A—right hand 53	A—left hand 54
B—right hand 52	B—left hand 60

The palm formulæ are as follows:

<i>Left Hands.</i>	<i>Right Hands.</i>
A—(11.7.7. 3) A.O.O.O.D.	A—(11.9.7 5') A.O.M.O.D.
B—(9.8.5".5') A/B.O.O.O.D.	B—(11.8.7.5') O.O.M.L.O.

In several respects there is a little more resemblance between right palm and fingers of the two than to their respective lefts, but this does not extend to details. On the whole these two girls make an entirely different impression. One has a rather pleasing, happy expression, the other a sullen, lowering expression. The fact that B is ambidextrous in both finger and wrist tapping suggests that she might be the left-hand component of a monozygotic twin pair, but there are too many differences between them to permit such a diagnosis.

Case 74.—These girls have many traits in common, but show also some extreme differences. A's height is 59 inches; B's is 53¾ inches. A's weight was 70½ pounds; B's 66½ pounds. A's head width is 14.1 mm.; B's 13.5 mm. A's head length is 17.6 mm.; B's is 17.1 mm. Hair color, texture and crown whorl same in both. Eye color of both a gray brown, but A's eyes are distinctly grayer and B's browner. B's ears are distinctly larger and wider although her head is considerably smaller. A's eyes are wider spaced than B's. A's nose is larger, longer and different in shape. B's teeth are crowded and overlap in front, while A's are straight.

Finger print formulæ:

<i>Left Hands.</i>	<i>Right Hands.</i>
1, 2, 3, 4, 5	
A—W, R, R, U, U	: A—W, R, U, U, U
B—U, R, U, U, U	: B—U, A, A, U, U

Quantitative values of finger patterns:

A—right hand 44	A—left hand 30
B—right hand 27	B—left hand 26



Palm formulæ:

<i>Left Hands</i>			<i>Right Hands.</i>	
A—(11.7.7.3)	O.O.O.O.D.	:	A—(11.9.7.3)	O.O.O.L.D.
B—(11.7.7.3)	O.O.O.O.O.	:	B—(11.7.7.3)	A.O.O.O.O.

Here again the palm formulæ suggests a closer resemblance than actually exists, in that we have the same pattern for the two left hands, but the two palms of B also have the same pattern and are far more similar in detail. Nowhere is there stronger cross resemblance than internal resemblance. On the whole there can be no doubt that these are fraternal twins.

Apart from these three cases there are no decidedly similar twins among the fifty-two pairs in our collection. Twenty pairs are designated as "slightly similar" fraternal twins and the remaining twenty-nine cases are designated as "unlike" fraternal twins. The slightly similar fraternal twins show merely the degree of resemblance common among siblings, while the unlike fraternal twins seem to show hardly as much resemblance as do average siblings. Even the three cases of decidedly similar fraternal twins, except for their identity in age, are no more alike than are occasional siblings. On the whole then, there seems to be nothing about these fifty-two cases out of accord with their classification as fraternal, or dizygotic, twins. Hence there is now no ground for doubting the validity of our classification of the one hundred two pairs of twins used in this study, into the two categories; monozygotic and dizygotic.

SUMMARY.

1. The original objective of these studies was the study of the rôles of heredity and environment in determining mental capacities of various sorts.
2. The first essential was to learn how to diagnose with certainty the two types of twins, monozygotic and dizygotic.
3. Only about 25 per cent. of all twins are monozygotic. Collections that depart widely from this figure have probably been incorrectly diagnosed.
4. Only about 42 per cent. of same-sexed twins are monozygotic.
5. The method of diagnosis used in this study combines the best

features of the methods of Dahlberg, Siemens, and Komai. Certain refinements of technique are added, the details of which are explained in the text.

6. Out of a collection of one hundred two pairs of same-sexed twins, only six pairs caused any difficulty, three of which are now classified as monozygotic and three as dizygotic.

7. The details concerning the three "similar fraternal" are presented and the reasons for their diagnosis as dizygotic twins are given.

8. The result is that we have now a collection of fifty pairs of monozygotic and fifty-two pairs of dizygotic same-sexed twins accurately diagnosed. These are to be used for further biological and psychological study.

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STUDIES OF HUMAN TWINS.

II. ASYMMETRY REVERSAL, OF MIRROR IMAGING IN IDENTICAL TWINS.

H. H. NEWMAN,

INTRODUCTION.

One of the most striking phenomena observed among monozygotic twins is that of the reversal of asymmetry between the individuals of a pair. Among armadillo quadruplets the present writer (Newman, 1916) found numerous instances in which a band or scute doubling occurred on the left side of one twin and on the right side of the other. Such cases call to mind the fact that in human double monsters (Siamese twins) *situs inversus viscerum* occurs in many cases. The same type of asymmetry reversal was noted by Swett and by Morrill in double-headed fish embryos. In separate identical twins in man it has been noted that the incidence of left-handedness in one twin of a pair is very much greater than among fraternal twins or in the general population of single individuals. Asymmetry reversal in the direction of crown whorl of the head hair seems to have about the same incidence in monozygotic twins, dizygotic twins, and single individuals as has left-handedness. These two expressions of asymmetry have been studied intensively in the present investigation and their significance will be discussed in some detail later.

HANDEDNESS AN EXPRESSION OF ASYMMETRY.

As an introduction to this study it seems well to examine the phenomena of handedness as it is found among human beings. In the first place, there are two distinct kinds of handedness: that which is genetically determined and that which is the result of twinning and therefore epigenetically determined.

Genetic handedness is evidently transmitted in such a way that any given zygote will give rise, when no twinning occurs, to a right-handed or left-handed single individual. There seems, however, to be varying degrees of right- or left-handedness. The majority of individuals, apparently about eighty per cent.

of single individuals, are definitely right-handed; about four per cent. definitely left-handed, and the remaining sixteen per cent. partially left-handed or ambidextrous. The incidence of right- and left-handedness is about what one would expect if right-handedness is a dominant Mendelian unit character and left-handedness recessive. The ambidextrous individuals and those showing lesser degrees of left-handedness may be heterozygous individuals in which the dominance of right-handedness is incomplete.

The other type of left-handedness, quite different in origin and heritability, is that which results epigenetically as the result of the twinning. Such left-handedness, being a somatic modification would not be hereditary: it would be merely an expression of asymmetry reversal due to the development of a whole individual from a half embryo which had already become more or less differentiated in a left-handed direction before the separation into twins has taken place.

Thus in genetic right-handed embryos which undergo twinning after some asymmetry has been established, the left-hand half embryo would be the superior one and would give rise to a right-handed individual, since right-handed superiority is due to left-sided superiority in the brain. Conversely, in a genetic left-handed embryo, the right side would be superior and the left side the inferior side, in which case the left-handed individual would retain the genetic asymmetry and the right-handed individual would exhibit asymmetry reversal.

In embryos genetically ambidextrous the right and left sides would be equal and would produce twins both of whom would be ambidextrous.

PREVIOUS DATA ON HANDEDNESS IN TWINS.

A good deal of attention has been paid by various authors to the peculiar incidence of left-handedness in twins. Siemens (1924) found in thirty-seven pairs of identical twins twenty-six cases both right-handed, ten cases in which one was right-handed and the other left-handed, and one case where both were left-handed. In a later paper the same writer reported on a larger number of identical twins (the total number not given) in which

there were twenty-one cases where one was right- and the other left-handed and three cases where both were left-handed.

Weitz (1924) found among eighteen pairs of identical twins, seven pairs composed of a right- and a left-hander, ten pairs both right-handed, and one pair both left-handed.

Dahlberg (1926) reports for sixty-nine pairs of identicals fifty-three pairs both right-handed, twelve pairs one left-handed, and four pairs both left-handed. Adding the three sets of cases together, we have one hundred and twenty-four cases of identical twins divided as follows:

89 pairs, both right-handed,	71.8 per cent.
29 pairs, one left-handed,	23.4 per cent.
6 pairs, both left-handed,	4.8 per cent.

Dahlberg has also studied the incidence of left-handedness in one hundred and twenty-eight pairs of dizygotic twins. The following figures indicate his results:

111 pairs, both right-handed,	86.7 per cent.
16 pairs, one left-handed	12.5 per cent.
1 pair, both left-handed,	0.8 per cent.

It will be seen that the incidence of left-handedness among identical twins is over twice as great as among fraternal, or four times as great in proportion to the number of zygotes involved, for a pair of identical twins involves only one zygote. Even among fraternal twins, the incidence of left-handedness is relatively high as compared with the general population, which is reported by Jones (1918) to be about four per cent. Jones' estimate, however, is probably much too low and takes account of only the most complete cases of left-handedness.

Lauterbach (1925) reports among fifty-seven same-sexed twins (not distinguished as to monozygotic or dizygotic origin) twenty pairs in which one was left-handed, about 35 per cent. of all cases. This is a higher incidence of left-handedness than any previously reported, especially when it is taken into consideration that the group examined consists of both identical and fraternal twins.

The most recent data on handedness in twins is furnished by Verschuer (1927). He found one or more left-handed individuals in 26.8 per cent. of seventy-nine pairs of identical twins and

in 26.3 per cent. of the thirty-eight pairs of fraternal twins. They were distributed as follows:

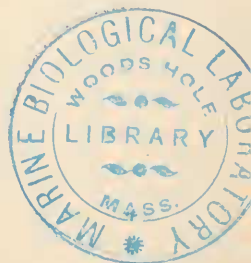
<i>Identical Twins.</i>	<i>Fraternal Twins.</i>
58 both right-handed.	28 both right-handed.
15 one right- the other left-handed.	10 one right- the other left-handed.
5 both left-handed.	0 both left-handed.
1 one right-handed, the other ambidextrous.	

The percentage is rather low as compared with those of others, particularly those of Lauterbach and the present writer, but the difference is probably due to the inclusion of only the cases of complete left-handedness. The percentage of pairs showing left-handedness among fraternal twins is exceptionally high and not in accord with the findings of others. Possibly the relatively small number of cases may be the cause of this discrepancy. Even more probable, it seems to me, is the inclusion among fraternal twins of a few of the least similar identical twins among whom left-handedness is common.

CRITERIA OF HANDEDNESS.

It is by no means a simple matter to diagnose left-handedness. There are many cases, of course, where the twins are (or were at an earlier period) obvious left-handers, but there are also many cases where congenital left-handedness is obscured by training the right hand and suppressing the left. Such cases often result in a sort of ambidexterity in ordinary manipulations. In our work we have used as a test of handedness speed in tapping with wrist and fingers. In all cases of complete left-handedness the tapping tests confirm the left-handed diagnosis. It appears to be safe then to use the tapping tests to reveal native left-handedness obscured by right-hand training or various degrees of partial left-handedness.

A good many cases of partial left-handedness were revealed by tapping tests. Among identical twins, in addition to the eleven pairs showing complete left-handedness, there were thirteen pairs (both of whom considered themselves right-handers) in which some degree of left-handed superiority was revealed in one or both members of the pair. In three of these pairs both members



were shown to be partially left-handed, and in two pairs both members were definitely ambidextrous.

Among fraternal twins, in addition to six pairs in which one individual was completely left-handed, there were five pairs in which one individual was partially left-handed, two pairs in which both were partially left-handed, and two pairs in which one individual was right-handed and the other ambidextrous.

Assuming that all these cases represent grades of left-handedness we have added to the seventeen pairs showing complete left-handedness twenty-two pairs showing partial left-handedness, a total of thirty-nine out of one hundred pairs in which some degree of left-handedness appears in one or both members of a pair. This high percentage would be much like that found by Lauterbach (35 per cent.) if we omitted the cases of ambidextrality.

CROWN WHORL AN EXPRESSION OF ASYMMETRY.

As is well known, the head hair at the crown twists or whorls in either a clockwise or a counter-clockwise direction. The great majority of individuals show clockwise hair-whorl, and therefore clockwise asymmetry may be considered as the normal and counter-clockwise asymmetry as the reversed asymmetry. Various writers have called attention to sporadic instances of reversed crown whorl, and a few cases have been described for identical twins.

Only one writer, however, has thus far made a systematic study of crown whorl in twins. Lauterbach (1925) in a study of resemblances and differences in twins has presented some very interesting data. Out of fifty-seven pairs of same-sexed twins there occurred fifteen pairs in which one or both twins showed counter-clockwise hair-whorl. In one of these cases both twins were counter-clockwise. This means that about twenty-six per cent. of the pairs of same-sexed twins showed asymmetry reversal in hair-whorl. In addition to these, there were six cases showing double crown in which one half of the whorl has a clockwise and the other a counter-clockwise direction. These cases are possibly comparable to ambidextrality in handedness and should probably be listed as a form of asymmetry reversal. Adding these six cases, the percentage of pairs showing more or less reversed hair-whorl among same-sexed twins comes to nearly

37 per cent., not unlike the percentage of left-handedness in the same set of twins, which was 35 per cent.

In our own collection of one hundred pairs of same-sexed twins there are in all ninety-five pairs in which it was possible to determine the hair-whorl. In five pairs (three identicals and two fraternal) the kinky or closely matted character of the hair rendered diagnosis of hair-whorl extremely difficult or impossible. Among the identicals there were twenty pairs showing some form of asymmetry reversal in crown-whorl. In fifteen pairs one twin showed clockwise and the other counter-clockwise whorl, in three pairs both twins were counter-clockwise, and in one pair one twin had a double crown and the other a clockwise whorl. The remaining twenty-seven diagnosed pairs showed clockwise hair whorl in both twins.

Among fraternal twins there were but five pairs having any form of asymmetry reversal in hair-whorl. In four of these pairs, one twin was counter-clockwise, and in one pair one twin had a double crown and the other a clockwise whorl.

As in the case of handedness, there are doubtless instances of incomplete asymmetry reversal that are not recognizable. Probably some of the crowns diagnosed as slightly clockwise or indefinite may be cases of partial asymmetry reversal.

Crown-whorl has one advantage over handedness as a criterion of reversal of asymmetry in that it is not subject to modification by training and is therefore a somewhat surer sign of asymmetry reversal than is left-handedness.

THE RELATION BETWEEN HANDEDNESS AND CROWN WHORL.

In only ten pairs of our identical twins do we find reversed asymmetry of any sort (either left-handedness, counter-clockwise hair-whorl, or both) in both twins of a pair. In eight of these cases (73, 25, 23, 87, 43, 38, 7, 27) both twins of a pair are left-handed or both have counter-clockwise hair whorl. It would seem natural to assume that all such pairs have been derived from zygotes, genetically left-handed. But what shall we do with the other two cases (13 and 72) in which one twin of each pair is plainly left-handed and the other clearly counter-clockwise in hair whorl? Since both of these indications are valid

criteria of reversed asymmetry there seems no escape from the conclusion that these two pairs also are derived from genetically left-handed zygotes.

THE INCIDENCE OF ASYMMETRY REVERSAL IN OUR OWN COLLECTION OF TWINS.

When the present study began, the writer was keenly on the lookout for evidences of asymmetry reversal in identical twins. The expectation was that the more strikingly identical the twins were, the more evidence of asymmetry reversal would be present. Before the study was half over it seemed certain that this expectation was not to be realized. In fact, the very opposite of this appeared to be true, namely, that the least evidence of asymmetry reversal appears among those twins that are practically indistinguishable, while the twins that are less nearly identical show the most evidence of reversal of asymmetry.

In order to test out this conception, the writer tried to arrange the fifty pairs of identical twins in the order of their closeness of resemblance, including resemblances in features, height, weight, headsize, finger prints and palm prints. After this was done, Mrs. Blythe Mitchell, the one who has had the most intimate and prolonged acquaintance with the twins, was asked to rearrange the cases according to her impression as to the degrees of identity. On the whole, there was a very close agreement, no case being changed more than a few places up or down in the series. Using the photographs, we worked over all cases together and arrived at the arrangement shown in Table I., which is not intended to be exact, place for place, but certainly represents a real grouping in the sense that the first five pairs are more similar than the second five, the second five than the third, the ninth five than the tenth. Within groups of five the order might be more or less shifted as the criteria of resemblance are not entirely objective, but depend to a large extent upon one's judgment of degrees of facial resemblance. In the following table asymmetries in handedness, crown-whorl, and head dimensions are given for the fifty pairs arranged in fives, beginning with the most alike and ending with the least alike. In this table *R* and *L* indicate definite right- and left-handedness, *l* indicates partial left-handedness,

A indicates ambidextrality; + indicates clockwise, or the common type of hair-whorl; and — indicates counter-clockwise hair-whorl. Double hair-whorls are indicated by (+ —).

TABLE I.

Serial No.	Twin.	Sex.	Handedness.	Crown Whorl.	Head Length.	Size Breadth.	Remarks.
62	A B	♂ ♂	R R	+ +	17.4 17.2	15.5 15.2	
98	A B	♀ ♀	R R	+ +	17.7 17.5	13.9 14.1	
63	A B	♂ ♂	R R	+ +	18.2 18.1	14.3 13.9	
40	A B	♂ ♂	R R	(+ —) +	17.85 18.1	15.0 15.5	
3	A B	♂ ♂	R R	? ?	20.0 19.7	14.75 14.3	Negroes. Crown-whorl could not be made out.
9	A B	♀ ♀	R R	— +	17.0 17.7	13.6 13.7	A shows partial asymmetry reversal in crown.
80	A B	♀ ♀	R R	+ +	18.7 18.1	13.5 13.2	B left-handed in wrist tapping.
67	A B	♂ ♂	R L	+ +	18.9 18.7	14.6 14.1	
55	A B	♂ ♂	R R	+ +	19.2 19.1	14.8 14.4	
35	A B	♂ ♂	R R	— +	18.55 18.55	15.0 15.1	A shows partial asymmetry reversal in crown.
96	A B	♂ ♂	R R	— +	18.10 18.15	13.9 13.9	A, incompletely reversed crown; left-handed in finger tapping. B, completely reversed in crown; nearly ambidextrous.
* 73	A B	♀ ♀	L A	— —	17.1 17.5	13.35 13.5	
102	A B	♀ ♀	R R	— +	17.8 18.1	15.0 15.0	
* 25	A B	♂ ♂	R R	— —	18.3 17.9	14.2 13.9	
30	A B	♀ ♀	R R	+ +	18.8 18.7	13.7 13.55	

TABLE I. (*Continued.*)

Serial No.	Twin.	Sex.	Handedness.	Crown Whorl.	Head Length.	Size Breadth.	Remarks.
* 23	A B	♀ ♀	A A	+ +	16.2 16.1	14.25 14.0	A more left-handed than B.
94	A B	♀ ♀	R L	+ +	18.4 18.15	13.5 13.2	
68	A B	♀ ♀	R R	- +	17.45 17.5	14.25 14.4	
49	A B	♀ ♀	l R	- +	18.0 18.2	14.6 14.1	
* 13	A B	♀ ♀	R L	- +	17.0 17.0	14.5 14.6	
78	A B	♂ ♂	L R	+ +	18.45 18.40	14.7 14.7	A more decidedly left-handed. Bats left-handed naturally. Left-handed in finger tapping. Left in both wrist and finger tapping. Left in finger tapping only.
* 87	A B	♂ ♂	A A	- +	19.95 19.7	13.9 14.0	
* 43	A B	♂ ♂	l l	- +	19.6 19.1	15.7 15.3	
* 38	A B	♀ ♀	l l	- -	19.4 19.1	15.5 15.5	
79	A B	♂ ♂	R L	+ +	17.35 18.1	13.9 13.3	
* 72	A B	♂ ♂	L R	+ -	19.15 19.25	14.5 15.0	
99	A B	♂ ♂	R R	+ +	18.2 18.25	14.5 14.5	
33	A B	♂ ♂	L R	+ +	18.05 18.05	14.9 15.25	
53	A B	♂ ♂	R L	+ +	19.0 18.8	13.9 13.4	
44	A B	♂ ♂	L R	+ +	18.5 19.5	14.3 13.9	
2	A B	♀ ♀	L R	? ?	17.45 17.95	14.45 14.35	
91	A B	♀ ♀	R R	+ +	18.0 17.9	13.8 13.9	
100	A B	♂ ♂	R R	+ +	17.65 17.2	13.6 14.0	

TABLE I. (*Continued.*)

Serial No.	Twin.	Sex.	Handedness.	Crown Whorl.	Head Length.	Size Breadth.	Remarks.
101	A B	♂ ♂	R L	+ +	18.6 18.9	15.5 15.5	
70	A B	♂ ♂	R L	+ +	18.5 18.1	15.6 15.3	
37	A B	♂ ♂	R l	+ +	19.15 19.35	14.75 14.65	Slightly left-handed in wrist tapping.
34	A B	♂ ♂	R l	+ +	18.8 19.0	14.8 14.8	Inclined to be ambidextrous.
28	A B	♀ ♀	R R	- +	16.8 17.7	13.8 13.9	Slightly left-handed in finger tapping.
* 7	A B	♂ ♂	l l	- +	17.3 17.6	15.7 15.5	Strongly left-handed in finger tapping.
6	A B	♀ ♀	R R	+ +	17.5 17.2	13.9 13.7	Strongly left-handed in finger tapping.
97	A B	♀ ♀	R R	+ +	17.85 18.0	16.0 16.0	
17	A B	♀ ♀	R R	+ +	17.85 17.75	14.45 14.65	
14	A B	♀ ♀	R R	- +	17.2 17.45	14.45 14.45	
15	A B	♂ ♂	R R	+ -	19.65 19.45	14.6 14.9	Both ambidextrous as babies.
69	A B	♂ ♂	R R	+ +	18.75 18.9	14.9 14.3	
24	A B	♂ ♂	R l	+ +	17.45 17.4	14.65 14.65	Slightly left-handed in playing marbles.
18	A B	♂ ♂	R R	+ +	18.6 18.8	13.8 13.8	
* 27	A B	♂ ♂	R l	- -	19.25 19.3	13.5 13.7	Slightly left-handed in wrist and finger tapping.
41	A B	♀ ♀	L R	? ?	17.26 17.6	15.2 15.6	Hair whorl could not be determined.
60	A B	♀ ♀	R l	+ +	17.6 17.5	14.1 14.15	Slightly left-handed in finger tapping.

HANDEDNESS IN RELATION TO DEGREES OF RESEMBLANCE.

In this table there are listed twelve pairs of twins one member of which is fully left-handed and, in addition to these, there are eleven cases that show partial left-handedness in one or both individuals of the pair. Besides the twenty-three cases showing some degree of left-handedness, there are two cases in which both members of the pair are classed as ambidextrous. Thus in exactly fifty per cent. of our pairs of identical twins there is some degree of left-handedness.

It is significant that the first case in the series to show complete left-handedness is seventeenth out of fifty. There are only two cases of partial left-handedness among the fifteen most strikingly similar set of twins, while some degree of left-handedness becomes the rule rather than the exception from the sixteenth to the end of the list.

CROWN WHORL IN RELATION TO DEGREES OF RESEMBLANCE.

The incidence of asymmetry reversal in crown hair whorl follows the same general lines as does left-handedness. In the first ten pairs there is but one case (No. 63, in third place) that shows true counter-clockwise hair-whorl. Two other cases (No. 9, in sixth place, and No. 35, in tenth place) show a mixed hair-whorl partly clockwise and partly counter-clockwise. There is also one case of a double hair-whorl, one whorl being clockwise, the other counter-clockwise (No. 40, in fourth place). The most frequent incidence of counter-clockwise hair-whorl in one twin occurs among the middle grade twins, neither the most alike or the most different. This is true also of left-handedness, and such a correspondence in the incidence of two forms of asymmetry reversal must have some real significance.

THE RELATION BETWEEN HANDEDNESS AND HEAD SIZE.

In the following study both left-handedness and counter-clockwise hair-whorl are taken to be equivalent criteria of either genetic or epigenetic reversal of asymmetry. For the present we shall omit from consideration the ten pairs of twins (Nos. 73, 25, 23, 13, 87, 43, 38, 72, 7 and 27) that were diagnosed as derived from zygotes genetically left-handed. These are starred in the list.

Before discussing the relation of head-size to handedness it should be said that there is undoubtedly some inaccuracy in the figures for head dimensions. Repeated measurements of the same head rarely give exactly the same result. Dahllberg has calculated that the average error in head measurements is about 0.5 mm. It seems probable that our own errors were at least as great as this, and probably greater. Hence differences of no more than 1 mm. may be ignored or considered as without significance.

Glance with me down the list of forty pairs of identical twins not previously diagnosed as derived from genetically left-handed zygotes. In all, there are seventeen pairs in which one twin may be classed as right-handed, the other left-handed, and in which there is a significant difference in head size. In thirteen of these pairs (63, 9, 67, 102, 68, 33, 53, 44, 2, 70, 28, 14 and 41) the right-handed individual, derived from the superior side of the embryo, has a distinctly larger head.

The four other cases (49, 79, 101 and 34) reverse this condition, the left-hander has the larger head, though case 34 is ambiguous in that one twin is slightly left-handed in tapping and the other nearly ambidextrous and may therefore belong with the list of ten diagnosed as derived from a left-handed zygote. The other three cases (49, 79 and 101) are valid exceptions. Let us consider these cases carefully. What would happen in the case of a genetically left-handed zygote if one of the twins underwent asymmetry reversal? Obviously the reversed twin would be a right-hander, and should have the smaller head. This interpretation appears to fit cases 49, 79 and 101. It would be strange if some cases such as these did not occur in view of the existence of genetically left-handed zygotes.

This hypothesis, that head size is correlated with handedness, may be checked still further by examining the ten pairs of twins diagnosed as derived from genetically left-handed zygotes. Of these, eight show a significant difference in head size. These eight cases deserve individual attention:

Pair No. 73.—This is a confusing case. Twin A shows left-handedness in finger tapping and has a partially reversed hair-whorl; twin B is practically ambidextrous in tapping and has a well-defined counter-clockwise hair-whorl, the only really posi-

tive indication of left-handedness present in the pair. This twin (B) has the larger head.

Pair No. 25.—In this pair both twins are right-handed and both have counter-clockwise hair-whorl. It is impossible to decide which of these has been derived from the superior side or whether they are derived from a right-handed or left-handed zygote, for the handedness and hair-whorl completely contradict each other.

Pair No. 23.—Both twins are ambidextrous, and both have clockwise hair-whorl. Twin A, with the larger head, is more nearly left-handed than B.

Pair No. 87.—Twin A, while ambidextrous, tends to be more left-handed than B, and has counter-clockwise hair-whorl; twin B is ambidextrous and has clockwise hair-whorl. Evidently A is the left-handed (superior) individual, and he has the larger head.

Pair No. 43.—Both twins are partly (probably natively) left-handed. Twin A has counter-clockwise hair-whorl, twin B clockwise. A, the more distinctly left-handed twin, has the larger head.

Pair No. 38.—Both twins are partially left-handed and both have counter-clockwise hair-whorl. A is left-handed in both wrist and finger tapping; B, only in finger tapping. A, the more left-handed, has the larger head.

Pair No. 72.—Twin A is strongly left-handed but has clockwise hair-whorl; twin B is right-handed but has counter-clockwise hair-whorl. It is impossible to say which individual should be diagnosed as from the superior side, since the two criteria seem to be of equal value. Of the two the reversed hair-whorl is somewhat safer as a criterion, and it happens that the twin (B) with the counter-clockwise hair-whorl has the larger head.

Pair No. 27.—Twin A is right-handed; B, slightly left-handed in wrist and finger tapping. Both have counter-clockwise hair-whorl. Twin B, the partially left-handed member of the pair, has the larger head.

All of these eight cases except pair 25, which is neutral, support the conclusion that the twin derived from the genetically superior side (the right side in these cases) of the embryo has the larger head.

One other class of cases remains to be dealt with, those in which a significant difference in head size exists without any complete

asymmetry reversal in handedness or hair-whorl. There are nine such pairs (62, 3, 80, 55, 30, 6, 97, 69, 18). In all but two of these cases (3, 55, 30, 6, 97, 69, 18) one twin was definitely more right-handed than the other and the more right-handed individual has the larger head in all pairs. In pairs 62 and 80, both twins are equally strongly right-handed and offer neutral evidence. Instead of weakening the general theory, then, all of these cases, where varying degrees of difference in right-handedness but no true left-handedness occur, tend strongly to support it. There is beyond question a strong correlation between handedness and head size. With very few exceptions indeed, the twin having the larger head shows evidence of having been derived from the genetically superior side of the embryo; from the left-hand side in twins derived from zygotes genetically destined to form right-handers, and from the right side of zygotes destined to form left-handers.

TWINNING AND THE ASYMMETRY MECHANISM.

The data just presented have given rise to a theory that seems to rationalize for the first time the peculiar incidence of reversal of asymmetry in twins. It is well known that in some groups of animals, notably those characterized by a striking degree of determinate cleavage, bilateral symmetry and asymmetry are established in the undivided zygote before or at the time of the first cleavage. In those forms, on the other hand, that show a strong tendency toward indeterminate cleavage, notably the vertebrates and echinoderms, symmetry and asymmetry are not definitely fixed until considerably later in development. The writer's work (Newman, 1924) on asymmetry reversal in the starfish indicates clearly that asymmetry is fixed before the time of gastrulation, for no reversal of asymmetry could be induced in embryos older than late blastulæ.

There are also indications among the vertebrates that asymmetry is established prior to or during gastrulation. Thus in the nine-banded armadillo, the only case of twinning among mammals where the stage at which twinning occurs is definitely known, it has been found that the first step in the twinning process usually precedes the period at which symmetry and asymmetry are es-

tablished and that the second step in twinning takes place during the process of the establishment of the axis of symmetry. By analogy, we may infer that twinning in man takes place in close association with, and possibly as an aberration of, the process of establishing and fixing the relations of symmetry and asymmetry in the embryo.

Now, since no biologic processes takes place with the same clock-like precision in all specimens, we may suppose that the twinning act in some cases is consummated during relatively early stages of the establishment of symmetry and asymmetry, and that in other cases it is established later. In the cases in which twinning occurs relatively late, the establishment of a single bilateral individual may have gone so far that complete twinning is impossible. This is probably the case in all partial twinning, resulting in conjoined twins and double monsters. In such twins one of the most striking features is the occurrence of profound reversal of asymmetry, as expressed in more or less complete *situs inversus viscerum*.

If then, we may assume that conjoined twins with the most extreme reversal of asymmetry in the inferior component, represents one end of the series of twins, it is natural to assume that the opposite end of the series is represented by cases in which twinning is consummated before any asymmetry is fixed. In such cases the twins would be derived from two equivalent primordia which had not yet been differentiated into right- and left-hand sides. When, later, asymmetry comes to be established in these two genetically equivalent and still undifferentiated embryos, it should follow the same course in both and each should develop the same asymmetry as the embryo would have done had it not undergone twinning. Thus, if the original embryo was genetically a right-hander, two right-handed twins should result; similarly, if the original embryo was genetically a left-hander, two left-handed twins should result—a condition not uncommon among twins, but hitherto unexplained. In such twins we would expect a high degree of same-sided asymmetry in such details as palm and finger prints, ear shape, dentition, handedness, hair-whorl, etc. Moreover, since the two twins are derived from two primordia that have not yet become differentiated as right- and

left-hand components, the two resulting twins would be expected to be very strikingly similar, more similar than would be twins separated after asymmetry had been more or less fixed in the embryo from which they are derived.

Thus the earlier twinning occurs with respect to the establishment of asymmetry, the more similar should be the resultant twins and the less should they show such evidences of reversal of asymmetry as left-handedness and counter-clockwise hair whorl. This explains why these criteria of asymmetry reversal are rarely present in the most strikingly similar twins and are increasingly common among identical twins that are less similar.

If this theory be sound, and there is much evidence in its favor, we have discovered another mechanism, not classifiable as environmental, that operates to make identical twins different. This factor, the asymmetry mechanism, may be the main, if not the only, factor responsible for observed differences between identical twins reared together. Consequently it would be quite unsafe to infer that any differences between such twins are due to differences in environment or in training. On the other hand, once we have established the average degree of difference between identical twins reared together, we should be able to use this as a base line in determining to what extent, in cases of identical twins reared apart, the differences in environment have operated to increase the physical or mental difference.

This theory goes far to explain why some, but not all, pairs of twins show left-handedness and counter-clockwise hair-whorl in one twin of a pair; why there should be occasional cases in which both twins of a pair are left-handed or have counter-clockwise hair-whorl; why there should be various degrees of incomplete asymmetry reversal as the result of separation of twins prior to complete establishment of asymmetry. The establishment of asymmetry is a progressive process and takes some time to become fully fixed. Hence we may expect to find that twinning early in the process will result in little if any signs of asymmetry reversal in one of the twins, and that twinning occurring late in the process will result in extensive reversal of asymmetry in one of the components.

In brief, this theory seems to clear up many if not all the

formerly baffling asymmetry situations found in twins. It lacks experimental confirmation, but this must be so from the nature of the material. Yet the data themselves almost speak out the theory of their own accord.

SUMMARY.

1. Reversal of asymmetry in monozygotic twins expresses itself in varying degrees, ranging from complete *situs inversus viscerum* in conjoined twins to left-handedness or counter-clockwise hair-whorl in separate twins.

2. There are two kinds of handedness: genetic and epigenetic. Genetic right- and left-handedness have about the incidence, respectively, of dominant and recessive allelomorphs. Epigenetic left-handedness (or in genetic left-handers, right-handedness) results from twinning, the inferior side having an asymmetry opposite to that of the superior side.

3. Arranging fifty pairs of identical twins in the order of their closeness of physical resemblance, it is found that there is very little evidence of asymmetry reversal among the most similar twins, while the less similar twins show a high degree of it.

4. Clockwise hair-whorl has about the same incidence as right-handedness, and counter-clockwise hair-whorl that of left-handedness.

5. Varying degrees of partial left-handedness and of ambidexterity are revealed by tapping tests.

6. Ten pairs of identical twins show asymmetry reversal in both members of a pair and are therefore diagnosed as derived from genetically "left-handed" zygotes; three pairs showing asymmetry reversal in but one twin should probably be classed as "left-handers"; the remaining thirty-seven pairs are believed to be derived from right-handed zygotes.

7. There is a very close correlation between head size and handedness. The twin derived from the superior side of the embryo nearly always has a significantly larger head.

8. The reason why many but not all identical twins show asymmetry reversal in one twin is that the epigenetic establishment of asymmetry takes place sometimes before and sometimes after twinning. If it takes place before twinning the twins will show

a high degree of asymmetry reversal; if it takes place after the twinning the twins will both show the same asymmetry and be in other respects more alike than when the establishment of asymmetry precedes twinning; if it takes place during the twinning process the twins will show varying degrees of asymmetry reversal in one individual and varying degrees of close resemblance in physical and mental characters.

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BIOLOGICAL BULLETIN

SEX DIFFERENTIATION IN GONADS DEVELOPED FROM TRANSPLANTS OF THE INTERMEDIATE MESODERM OF *AMBLYSTOMA*.

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INTRODUCTORY AND HISTORICAL.

From his studies on parabiotic twins in *Amblystoma punctatum* Burns ('25) is led to the conclusion that in this species there may occur a complete reversal of sex previous to the period of sex-differentiation. Embryos joined in pairs in early stages should, by the laws of chance, be combined in the proportion of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀. Instead of this expected ratio, Burns obtains exclusively one-sexed pairs, in the proportion of 44 ♂♂ to 36 ♀♀. This result he is inclined to interpret as a 1 : 1 ratio. Having no evidence that the two-sexed pairs had been eliminated through selective mortality, Burns postulates that half the pairs reared must have been, originally, ♀♂ combinations; in these pairs, from a condition of near-equilibrium as regards sex, one or the other sex, he assumes, had eventually gained the ascendancy, so that at sex-differentiation the gonads of the two members of the pair were identical. Since the sex-ratio found was approximately 1 ♂♂ : 1 ♀♀, Burns infers that there can be no prepotency constantly favoring either male or female, since in this event a 3 : 1 ratio favoring the prepotent sex would be expected.

The more recent studies of Witschi ('27) on frog embryos joined in parabiosis show that in these amphibia the early sex reversal assumed by Burns does not occur. Witschi finds in 56

pairs the following combinations: ♂♂, 16 pairs; ♂♀, 17 pairs, with 7 of the females undergoing sex-reversal; ♀♂, 10 pairs, with 4 of the females undergoing sex-reversal; ♀♀, 13 pairs. This approximates very closely the expected ratio of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀, and shows conclusively that there could have been no sex-reversal previous to the time of sex-differentiation. From the fact that in many of the two-sexed pairs the females were found undergoing sex-reversal, while a female united with a male undergoing reversal was never found, Witschi concludes that the male is always dominant in the sex-reversal which finally occurs. Though he believes that the independent sex-differentiation in the individuals of genetically two-sexed pairs favors the theory of localized sex-differentiators ("lokalisierte Innenfaktoren," probably comparable to Spemann's "Organisatoren"), he states that in the later sex-reversal of the female of the pair, "the coöperation of hormones is not improbable."

The method of parabiosis used by Burns and Witschi has certain obvious disadvantages. If used with a species in which the zygotic sex-determination can be completely reversed previous to sex-differentiation, as is possibly the case in *Amblystoma*, there can be no certainty regarding the original state of any one-sexed pair examined after sex-differentiation has taken place. In drawing conclusions as to the occurrence of sex-reversal in these one-sexed pairs, one must depend entirely upon the sex-ratio obtained. Further, if the death rate among pairs joined in parabiosis is high, the possibility of a selective mortality cannot be entirely eliminated, even though evidence in favor of it may be scanty or lacking. Hence there is no absolute proof that the one-sexed pairs found at sex-differentiation were not all of this character genetically at the time they were joined; the proof of sex-reversal, therefore, remains inconclusive.

The method of parabiosis is relatively advantageous if used with a species in which an early reversal of sex does not occur (as *Rana sylvatica*; Witschi, '27). In such a species, pairs preserved at a suitable period in development would show the actual progress of sex-reversal in one member of the pair. If, however, the reversal becomes complete, all pairs killed at later periods would be found to be one-sexed. Although sex-reversal could be confidently asserted for a species of this type as a result

of the disappearance of the two-sexed condition observable in younger pairs, the identity of any of the originally mixed pairs could be established in adult animals only with great difficulty if at all.

In the spring of 1926 the writer undertook to transplant the intermediate mesoderm of *Amblystoma* from one embryo to a latero-ventral site in another in order to determine the fate of the primordial germ cells included in such grafts. Among the seven embryos surviving the implantation was one in which at forty-four days after operation the germ cells of the graft were found to have given rise to a gonad of considerable size (Humphrey, '27). This suggested the possibility that such grafts, if allowed to develop until after the period of sex-differentiation of the host, might be found to contain gonads which had likewise undergone sex-differentiation. The donor serving as the source of the transplant, and the host into which it was engrafted, though selected at random long before sex-differentiation had occurred, must in many cases be unlike in sex. Since the donor could be reared, its sex could be determined from the gonad it possessed, and since donor and host were not joined, the sex-differentiation in either could not be influenced by the other, except in so far as the graft might be able to modify the sex-differentiation of the host. If, then, after sexual differentiation the gonad of the graft were found to agree in type with that of the host regardless of the sex of the donor, the fact of an early sex-reversal would be established beyond question. If, on the other, hand, the gonad of any graft differed in type from that of the host, agreeing with that of the donor, it would show conclusively that sex-reversal previous to sex-differentiation had not occurred. By the method of grafting, therefore, it seemed possible to obviate certain difficulties inherent in the method of parabiosis. The donor furnishing the graft would undergo sex-differentiation according to the factors present in the egg at fertilization; its gonad could be compared directly with the gonad developed in a transplant removed during the germ-layer stage and grown in a host of the opposite sex. Conclusions as to the occurrence of sex-reversal, therefore, could be drawn from comparison of structures rather than by reasoning

from sex ratios in which the factor of selective mortality might possibly be involved.

MATERIAL AND METHODS.

The removal of the intermediate mesoderm (preprimordia of gonad and mesonephros) of *Amblystoma* and its implantation into another embryo is a relatively simple operation, the technique for which has been outlined elsewhere (Humphrey, '27). During the operating season of 1927, 180 such implantations were carried out. The graft always included a large part of that region of the intermediate mesoderm in which it had been found that primordial germ cells develop (*i.e.*, the territory of the seventh to the seventeenth somites, approximately); in addition it included parts of the adjacent axial and lateral mesoderm, together with the overlying ectoderm.

Following operation, the host receiving the transplant and the donor furnishing it were reared to the age of fifty days or over—*i.e.*, until after the beginning of morphological sex-differentiation. At autopsy of the host the graft derivatives were found, as a rule, attached to the inside of the ventral or lateral body wall. In the donor, at autopsy, the gonad was always very small or entirely lacking on the right, the side from which the transplant was invariably taken in the embryo.

RESULTS.

Of 180 pairs (donor and host) only 49 or 27 per cent. of the total, were reared to the age of 50 days or over. This, however, does not indicate an actual mortality of 73 per cent. in the grafted animals, since 25 additional hosts were reared to the age of 50 days or more, although the donors which had furnished them transplants had died in early stages of development. Several hosts were also killed before reaching the age of 50 days, in order to study the development of the gonad and other structures in the graft; these hosts were always those of pairs from which the donor had already died from operative injury or other causes. In all, 74 grafts were recovered after sex of the host had become distinguishable. Of these grafts, 40 contained a gonad, the sex of which was determinable with a fair degree of certainty in 33 cases. In the remaining grafts the

gonad was small with few germ cells and no features permitting it to be classified as either ovary or testis.

Unfortunately for this study, the majority of the gonads which developed were in homoplastic transplants in *Amblystoma jeffersonianum*. In this species, instead of the expected 1 : 1 sex-ratio, the animals reared in the laboratory in 1927 were in the proportion of 56 females to 19 males, essentially a ratio of 3 : 1. As a result of the predominance of females, donor and host were both of this sex in an excessive number of cases. In only two instances were donor and host unlike in sex and in these, unfortunately, the gonad of the graft was in each case of somewhat atypical structure due to unfavorable environmental factors.

To the writer's knowledge a sex-ratio such as the one here reported for *Amblystoma jeffersonianum* has not been previously recorded for this species. Whether it is to be explained on the basis of a selective mortality among operated animals, or whether it is due to an induced reversal of sex in certain males resulting from nutritive disturbance or other alteration of environmental factors, or whether an excess of females is a normal condition in this species or at least in its local strain, cannot be positively stated. It is worthy of note that in *Amblystoma maculatum* (*punctatum*) reared in the laboratory under identical conditions and after similar operative procedure, the sex-ratio is apparently quite normal. The collection of large numbers of *A. jeffersonianum* larvæ from local ponds and a study of their sex-ratio has not been possible. The few specimens picked up near ponds after metamorphosis have been found to be females in the great majority of cases.

A second feature of interest noted particularly in this species is the occurrence of spermatocyte stages in the testes of males 60 to 80 days of age. This cannot be due to the presence of a graft furnished by a female, since spermatocytes are no more frequent in hosts than in donors. Though Burns ('25) makes no mention of spermatocytes in *A. maculatum* of similar age, the writer has encountered such stages occasionally in this species as well as in *A. jeffersonianum*. In the latter, however, they occur in a higher percentage of the males examined, and usually in greater numbers than in *A. maculatum*. In neither species, were the spermatocytes found in stages later than the pachytene

condition of the heterotypic prophase. Though the presence of heterotypic prophases in males renders these stages of little value as a criterion of sex when considered alone, it may be noted that their number in the male is small as compared with the number of other germ cells, and that they were not found in the diplotene or later stages characteristic of the oöcytes of amphibian females.

Of 56 *Amblystoma maculatum* reared in the laboratory in 1927 30 were females and 26 males. These numbers give an approximation of the expected 1 : 1 ratio. In this species, however, the majority of the transplants used were furnished by very young donors (stages 21 to 25¹) and but few gonads developed. In only two cases in which the sex of the donor was known to differ from that of the host was a gonad present in the graft. In one of these two the gonad was small and of the indifferent type, while in the second it was of a type combining features of both ovary and testis.

From the above it may be seen that relatively little evidence bearing upon the problem of sex-reversal was obtainable from grafts the donors of which had survived to sex-differentiation. But in several cases in which the donor had died before reaching this period, the transplant furnished by it was found to contain a gonad differing in sex type from that of the host in which the graft had developed. In these cases it would appear that donor and host must have been unlike in sex, but that the gonad of the graft had differentiated in a fashion determined by the organization of the transplant previous to its isolation from the donor. These cases may now be described in some detail.

No. 211.—Transplant from *A. jeffersonianum* of stage 29 implanted in *A. maculatum* of stage 25. The donor died 18 days after operation. The host, killed 61 days after operation, proved to be a female. A section of the ovary is shown in Fig. 1. The central ovarian cavity is well developed, and the germ cells are peripheral in position. Their nuclei are largely in the heterotypic prophase stages characteristic of the early urodele ovary, although few in number or lacking in the testis, as a rule, until a much later period of development. The graft removed from this host

¹ The stages referred to throughout this paper are those of Harrison's series of standard stages.

included a fairly large gonad of testicular type (see Fig. 2). No central cavity is present. The germ cells are somewhat uniformly scattered through the organ, intermingled with numerous smaller cells which constitute the 'sex cords' (anlagen of duct system), and the stromal and sustentacular elements of the testis. The germ cells are all in spermatogonial stages; heterotypic prophases are entirely lacking.

No. 284.—Transplant from *A. maculatum* of stage 30 implanted in host of same species and stage. The donor was killed by the host 37 days after operation. The host, autopsied 58 days after implantation of the graft, is a female. Although sex-differentiation had but recently occurred, the ovary has the characteristic central cavity and peripheral oöcytes with nuclei in heterotypic prophase (see Fig. 3). The gonad found in the transplant is a pear-shaped testis attached by a stalk to the surface of the graft mesonephros. It lacks the central cavity characteristic of the ovary, and shows the more uniform distribution of germ cells typical of the young testis (see Fig. 4). No heterotypic prophases are present, all germ cells being in spermatogonial stages.

No. 244.—Transplant from *A. jeffersonianum* of stage 31, implanted in host of same species and stage. Donor presumably devoured by host at about 31 days after operation. The host, autopsied 61 days after operation, is a male; a section of one testis is shown in Fig. 5. As is frequently the case in males at this stage of development, the testes of this animal show a few cells in the spermatocyte stage, but the germ cells are distributed in the fashion characteristic of the testis, and no central cavity is present. For comparison with the testis of the host a section of the gonad of the graft is shown in Fig. 6. This gonad must be interpreted as an ovary in an early stage of sex-differentiation. Although no central cavity is yet present, the germ cells are arranged in a layer around the periphery of the gonad and are for the most part oöcytes in earlier stages of the heterotypic prophase. By comparison of Fig. 6 with Figs. 1 and 3 (ovaries of fairly early stages of differentiation) it will be readily appreciated that this graft gonad is ovarian in nature. The differences between it and the graft gonads of Figs. 2 and 4 (testes) are

clearly evident from the photographs, and need no further comment.

The three cases above described show clearly that a gonad developing in a graft need not agree in sex type with the gonad of the host. It may be logically inferred that in these three cases the sex-differentiation of the graft gonad was determined by the organization in the implanted mesoderm previous to its removal from the donor embryo.

In a few cases in which both donor and host lived until after sex-differentiation and were found to be of unlike sex, a gonad was present in the graft. These cases, however, are less satisfactory than the preceding, since the gonad of the graft is either in an early stage of sex-differentiation or is of atypical structure. Three such cases will now be described.

No. 207.—Transplant from *A. jeffersonianum* of stage 29 implanted in *A. maculatum* of stage 25. The host, killed 61 days after operation, is unquestionably a male, although a few germ cells in heterotypic prophase are found in one of the testes. A section of the testis is shown in Fig. 7. The donor, a female, was not killed until 78 days after operation. The gonad shown in Fig. 8 is therefore more advanced in development than the testis of Fig. 7. The gonad found in the graft is small and in an early stage of sex-differentiation. Although no central cavity is present, the germ cells tend to take a peripheral position. Of the 95 germ cells present, 38 are in early stages of the heterotypic prophase. Considering all its structural features, this gonad should be classed as an ovary. In the peripheral arrangement of its germ cells, and in the high proportion of these cells found in heterotypic prophase, it is clearly similar to the gonad of the donor rather than to that of the host.

No. 190.—Transplant from *A. jeffersonianum* of stage 33 implanted in host embryo of same age and species. The host, killed 64 days after operation, is a female (see ovary in Fig. 10). The donor, killed at the same age as the host, is a male (see Fig. 11). The gonad of the graft is atypical in structure in that an unusual amount of stroma is present, in the form of a mucous type of connective tissue (Fig. 12). It may nevertheless be classed as testis rather than ovary. The germ cells, though frequently included in the covering epithelium, are predominantly

scattered through the central part of the organ. No central cavity is present. Sex cords (anlagen of duct system of testis) are recognizable as groups or strands of smaller cells, in some sections extending a third or more of the length of the gonad. The germ cells are for the most part spermatogonial in type, only three or four of the several dozen present being in heterotypic prophase, and none of these having the characteristics of growing oöcytes. Though of atypical structure, this gonad cannot be considered as undergoing transformation from testis into ovary. Aside from the abundance of mucous connective tissue, its structural features are clearly similar to those of the testis in the donor. Atypical gonads of the same general appearance may develop in grafts from a male donor implanted in a male host. The peculiarities of structure exhibited are therefore due, probably, to the action of local environmental factors rather than to the activity of sex hormones secreted by the gonads of the host.

No. 188.—Transplant from *A. jeffersonianum* of stage 33 implanted in host of same age and species. Both donor and host were killed 64 days after operation. The host is a female, the donor a male (see Figs. 13 and 14). The gonad of the graft is an atypical structure difficult to classify (see Fig. 15). Neither typical ovarian cavity nor testicular duct system is recognizable. The germ cells are predominantly peripheral in location, although frequently scattered or in masses deeper within the stroma. In one instance a mass of germ cells lies in a cavity, with no apparent attachment to other tissues of the gonad; these cells show marked degenerative changes. The cells at the periphery of the gonad frequently exhibit a grouping or 'nesting' comparable to that of young oöcytes in a normal ovary. Though for the most part in heterotypic prophase (several hundred such cells must be present) these germ cells seem never to progress beyond the pachytene stage. If the gonad were actually ovarian, some few at least of these cells might be expected to pass through the diplotene stage and then enlarge as growing oöcytes. This has been found to occur in those atypical gonads which have developed in grafts from female donors. In this gonad, however, no growing oöcytes are present, numerous pyknotic and fragmenting nuclei indicating the degeneration of the germ cells

during the pachytene stage rather than their continued development.

While it might appear at first glance that the features exhibited by this gonad have resulted from the action of the hormones of the host, it is highly probable that many of its peculiarities are referable to the growth potentialities of the implanted tissue as modified through local environmental influences. The donor furnishing this particular transplant exhibits an unusual number of spermatocytes in its one (left) gonad. Four such cells may be recognized in the section shown in Fig. 14 (at left). Presumably the tissue implanted possessed the potentiality for developing a gonad in which unusual numbers of heterotypic prophases would have appeared precociously, even without an endocrine stimulus from a female host. As to local environmental conditions, it may be noted that the gonad was attached by a very delicate fold of tissue, and was apparently poorly vascularized. The latter condition alone would be unfavorable to the development of a gonad of normal histological structure.

In addition to the graft gonad above described (No. 188) two other specimens exhibit features which might possibly be interpreted as modifications due to the action of sex-differentiating hormones. In one of these the graft gonad consists of a central core of testicular character overlaid by a cortex ovarian in type. This structure resembles the modified testes described by Burns ('28) as resulting from the action of ovarian hormones. The position of this particular graft in the body of the host, however, is such that some of the primordial germ cells of the host may actually have entered into the make-up of the graft gonad. If this be the case, this structure must be regarded as a 'mosaic' gonad derived from two preprimordia of unlike sex-potentialities rather than as a testis undergoing sex-reversal due to the endocrine influence of a female host. It is significant that graft gonads developing in sites sufficiently far ventral to exclude the possibility of actual contribution of host germ cells generally show no indication of sex-reversal (see Figs. 2, 4, and 6).

Among those cases in which only the host survived until the period of sex-differentiation are seven in which the gonad of the graft agrees in type with those of the host. While a reversal of sex in these few cases cannot be positively excluded, it is

rendered exceedingly improbable by the fact that in five other cases the gonad of the graft is of opposite sex from those of the host. Examples of this latter group have already been described (Nos. 211, 284, and 244; Figs. 1 to 6).

DISCUSSION.

The outstanding feature of the results described in the preceding pages is the apparently independent sex-differentiation of the gonads which develop in grafts. Although in one or two cases such a gonad has been modified in a fashion suggesting an influence from sex hormones of the host, in no case is a complete early reversal of sex clearly indicated. So far as can be determined from cases in which the sex of the donor is known, the primary sex-differentiation in the gonad of the graft always proceeds in a fashion determined by the sex of the donor. In five cases in which the sex of the donor is not known, the gonad is of opposite sex from that of the host. In four of these cases, gonads with the features characteristic of a testis have differentiated in grafts implanted in female hosts, while in the fifth an ovary has developed in a graft implanted in a male.

It is difficult to reconcile these findings with the conclusions reached by Burns ('25) from his studies on the sex of parabiotic twins in *Amblystoma*. Burns finds that the sex of the two members of any pair is always the same. From this he is led to infer that complete reversal of sex has occurred in one member of all two-sexed pairs, such reversal being accomplished before sex differences in the gonads become morphologically distinguishable. He assumes that when embryos of unlike sex are joined in parabiosis there results a condition of close balance or unstable equilibrium, which is broken if one animal of the pair gains a slight advantage, presumably through earlier or more abundant output of sex-differentiating hormones. All hormones being mingled in the blood stream, and neither sex being constantly prepotent, either the male or the female hormone may become dominant. Such domination being established before the onset of morphological sex-differentiation, the phenomena of this period will be identical in the two members of any parabiotic combination, or essentially so. The twin which has undergone reversal thus differentiates directly without first exhibiting the

sexual characters to be expected from its genetic constitution. Under these conditions, a reversal cannot be detected by study of developmental stages of the gonads but must be inferred from the absence of two-sexed pairs after morphological differentiation has been completed, unless it be assumed that all such pairs have been eliminated through a selective mortality.

In discussing his results, Burns considers the possibility that a 'selective' mortality has operated to eliminate all heterogeneous (male-female) pairs, permitting only homogeneous pairs to survive. While this explanation cannot be positively rejected, Burns regards the occurrence of a selective mortality as highly improbable. Although the death rate among his operated animals is very high (about 77 per cent.), he believes that it is possible to explain it without postulating a physiological incompatibility of the sexes so profound as to induce the death of all two-sexed pairs. Witschi ('27) has demonstrated that no such incompatibility exists in the frog, since he finds the expected number of mixed pairs at metamorphosis of his parabiotic animals. If we assume that among Burns's experimental animals there was likewise no selective mortality eliminating mixed pairs, we are forced to conclude that parabiosis induces an early sex-reversal in one member of every two-sexed pairs.

If sex-reversal in parabiotic twins in *Amblystoma* be assumed to have occurred in the manner postulated by Burns, it would be logical to expect a reversal of sex in the gonad of a graft implanted in a host of opposite sex from that of the donor. The bulk of the transplant is small compared with the entire body of the host, and the gonad to which the graft gives rise is but a fraction of the size of the host's own gonads. Under these conditions there should exist no state of near-equilibrium as regards sex. If sex-differentiating hormones are produced previous to morphological sex-differentiation, those of the host should always, from their greater abundance, be able to dominate the differentiation of the gonad in the graft;² the latter, therefore, should always

² The gonad of the graft is often somewhat retarded in development as compared with those of the host, possibly, in some cases, because of inadequate nutrition. Such retardation of its development should favor modification of the graft gonad by the gonads of the host, assuming that sex differentiating hormones are poured into the circulation when the gonads reach a certain stage in their differentiation.

agree in type with the gonads of the host. Yet the gonad of a graft is clearly able to develop as ovary in a male host, or as testis in a female. In none of my animals could sex-reversal be demonstrated as having preceded the primary sex-differentiation.

Since the extent to which a hormone may modify an embryonic structure probably depends in part upon the period of development at which it is introduced and the time during which it is allowed to act, these conditioning factors may well be compared for parabiotic twin and graft.

In Burns's experiments, *Amblystoma* embryos were joined in parabiosis at about stage 28 of Harrison's series. In my own experiments many of the grafts were implanted at this or even earlier stages. In none of the cases considered in this paper was either donor or host more advanced in development than stage 34 at the time of operation. In neither the parabiotic twins at the time of union nor in the host receiving an implant has the blood yet begun circulation. While it is probable that the blood streams of embryos joined in parabiosis are in communication from the time the circulations of the two first become established, my observations indicate that the graft becomes vascularized at a correspondingly early period in its development. In short, the sex-modifying influence of the host upon the graft should be exerted fully as early as the influence of an embryo upon its parabiotic twin, assuming that this influence is mediated through the activity of substances transported by the blood.

As regards the actual time elapsing between operation and autopsy, the advantage appears to lie with the parabiotic twins. Burns states that among the pairs of his series even the best did not show sex-differentiation until seventy days, while the general average required considerably longer (eighty to ninety days) for sex to become clearly distinguishable. In my own animals sex was usually determinable without difficulty at fifty days after operation. The longer indifferent period in the parabiotic twins doubtless results chiefly from growth retardation due to difficulties in feeding. In any event, it greatly increases the period over which one animal is subjected to the influence of the other before morphological sex-differentiation occurs. Possibly in this prolonged indifferent period the physiological state of the gonads in one animal may be so altered through the influence

of its opposite-sexed twin that at the time morphological sex-differentiation finally occurs the gonads of the two animals differentiate in identical fashion. In my own experimental animals the shorter indifferent period may be insufficient to effect such a physiological reversal in the gonad of the graft, which in consequence differentiates as determined by the genetic constitution of the donor. In the parabiosis experiments of Witschi the indifferent period (in *Rana sylvatica*) is likewise short, which may possibly explain the fact that sex-reversal of the female follows rather than precedes the primary morphological differentiation of the gonad.

It is also possible that conditions attendant upon development of the graft may render it less subject to hormone influence from the host than is a parabiotic twin to the influence of its mate. Since the graft usually becomes well vascularized, however, it would seem that the nutritive materials and hormones of the host's blood stream should be as readily available for the gonad of the graft as for the host's own gonads. As has been stated before, sex-differentiating hormones of the host should be but little diluted by antagonistic hormones secreted in the graft. Moreover, the removal of the graft from its natural environment in the donor while in a germ-layer stage and its implantation into an essentially foreign situation should, if anything, disturb the action of local factors affecting sex-differentiation, and facilitate the modification of this process through hormones produced by the host. It would seem that in a graft the developing gonad has been removed from both the endocrine and environmental influence of the donor and subjected to the influence of the host in a far more complete fashion than the gonads of one parabiotic twin can be brought under the influence of the other embryo of the pair.

From comparison of the conditions acting upon parabiotic twin and graft, we may conclude that two, at least, possibly have significance in determining the difference in the results obtained. First, the greater time required for morphological sex-differentiation in parabiotic twins may permit an influence of one animal upon the other such as would not be possible in the case of a graft gonad differentiating in from half to two-thirds of the same period. Secondly, the fact that in one case (parabiosis)

the gonad has remained undisturbed in the organism, while in the other its preprimordium has been implanted in an ectopic situation in another individual, may possibly explain the different way in which it reacts preceding or during sex-differentiation.

The results obtained by the writer in *Amblystoma* are not without parallel from experimental work on other vertebrates. Willier ('27), from his study of the differentiation of chick gonads implanted in the chorio-allantoic membranes of either male or female hosts is led to the conclusion that "the course of sex-differentiation in the chick embryo is apparently not determined by the action of sexual hormones circulating in the blood stream." He believes that "hormonic sex-differentiating factors of the host embryo are either absent, or if present, they are ineffective in the modification of the engrafted sexual glands." Witschi ('27b) reaches similar conclusions from one of his latest studies on sex-differentiation in *Rana temporaria*. He finds that the implantation of a large graft of adult frog testis in tadpoles of this species does not "exert the least influence upon the larval and early post-larval development of the gonads." In both frog and chick, therefore, the indifferent gonads are found to undergo their primary sex-differentiation apparently unmodified by sex hormones from outside sources. In cattle, too, recent studies may be interpreted as showing that even when the chorions of two-sexed twins are fused at a very early period, the gonad of the female co-twin first begins to differentiate as an ovary, and only later undergoes modifications leading to the production of the characteristic free-martin gonad (Lillie, '23; Bissonnette, '28).

That the vertebrate ovary in situ may be modified in its development subsequent to its primary sex-differentiation is apparent from the studies of Lillie ('17) and others on the free-martin, or from the cases of sex-reversal in parabiotic frogs reported by Witschi ('27a). That these same gonads would have undergone a comparable modification if implanted as grafts in a host of the opposite sex has not been actually demonstrated. According to Willier, no modification of engrafted gonads of the chick is demonstrable after a period of nine days on the host embryo. It is conceivable, however, as Willier states, that the transplantation of the embryonic sexual glands into chicks after hatching might yield different results than when these same

glands are implanted on the membranes of embryonic hosts. Greenwood ('25) has reported the development of spermatic tubules in grafts of the left ovary taken from chicks two to four days after hatching and implanted in young chicks of the same age. It would appear probable, therefore, that isolation and implantation of a gonad (or its preprimordium) do not necessarily prevent the modification of that gonad through the action of sex-hormones of the host: *i.e.*, there remains possible an inhibition of growth, or an induction of growth, in those parts (as for example the medullary cords of the bird's ovary) which have retained their embryonic capacity to react in a specific fashion to growth stimuli.

The grafts described in this paper were in no case left implanted in the host for a period longer than seventy days. Although in none of the grafts recovered had the gonad undergone a complete reversal of sex previous to its primary differentiation, it is possible that in one or two cases it had undergone some slight modification which might be ascribed to the action of sex hormones of the host. Whether a complete reversal of sex might have occurred had the graft remained implanted for a longer period is problematic. From grafting experiments recently reported by Burns ('27) it is evident that sex-reversal in the gonads of *Amblystoma* is not complete even after periods of from fifty to seventy-six days in a host of the opposite sex. Burns transplanted gonads from larval stages, just before and just after the beginning of morphological sex-differentiation, into older larvæ in which sex-differentiation was more advanced. Since several of the grafts showed an admixture of the characteristics of the two sexes, it is possible that a complete reversal of sex might eventually have been effected.

Whether or not complete reversal of sex in *Amblystoma* may occur subsequent to morphological sex-differentiation, a reversal of sex preceding this period does not appear to be effected in gonads developed in grafts, when such grafts are implanted in an ectopic situation, such as the latero-ventral body wall. Whether implantation of the graft into its normal site would insure reversal of the gonad as postulated for animals joined in parabiosis still remains a question. The writer now has in progress an extensive series of experiments to test this point.

SUMMARY AND CONCLUSIONS.

1. An area of mesoderm which included the preprimordium of the gonad was transplanted from one *Amblystoma* embryo to another at stages 21 to 34. Such transplants, when taken from donors older than stage 25, gave rise to a gonad in a high percentage of cases. This gonad was ectopic in position, being attached to the inside of the lateral or ventral body wall, and was always far smaller than the normal gonads of the host.

2. Morphological sex-differentiation occurred in the grafts at from fifty to sixty days after implantation. All grafts were removed and fixed within seventy days. In several cases, gonads of testicular type were recovered from female hosts. In two cases gonads of ovarian type were found in grafts implanted in males.

3. In two cases in which donor and host were of opposite sex the gonad of the graft was modified in such fashion as to suggest an influence from sex hormones of the host. In no case, however, was the sex of the graft gonad completely reversed previous to the period of morphological sex-differentiation.

4. It may be concluded that gonads developed in ectopic grafts of the gonadal preprimordia undergo their primary morphological sex-differentiation according to the organization of the graft at the time of its removal from the donor.

5. If sex-differentiating hormones are produced by the host previous to morphological sex-differentiation, they are apparently incapable of bringing about reversal in the gonad of the graft. The possibility of reversal at a later stage of development is not excluded, since no grafts were allowed to develop for periods longer than seventy days.

6. The failure of the gonad in a graft to undergo sex-reversal previous to its morphological differentiation is in marked contrast to the complete reversal which appears to occur in parabiosis (cf. Burns '25).³

³ Studies completed while this paper was in press indicate that the graft ovaries of Figs. 6 and 9 possibly owe certain features of their structure to the action of the testicular hormones of the host. These studies show that the developing ovary is readily modified if subjected to the continued influence of a testis resident in the same host, and that one of the first perceptible indications of this modification is the absence of the characteristic central ovarian cavity. These studies will be reported in a separate paper.

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PLATE I. EXPLANATION OF FIGURES.

All figures on this plate are photomicrographs. Magnification 145 X.

FIG. 1. Ovary of host No. 211, *Amblystoma maculatum*, killed 61 days after implantation of graft at stage 29. The central ovarian cavity is well developed, and the germ cells peripheral to it are chiefly oöcytes in heterotypic prophase. Compare with Fig. 2.

FIG. 2. Testis of graft recovered from host No. 211. Note the absence of a central cavity. The germ cells are uniformly distributed, and none are in heterotypic prophase. Compare with the ovary of the host (Fig. 1). This testis was attached to the body wall by a slender stalk not included in this section.

FIG. 3. Ovary of host No. 284, *Amblystoma maculatum*, killed 50 days after implantation of graft at stage 30. Ovarian cavity, peripheral arrangement of germ cells, and abundance of heterotypic prophase stages, as in Fig. 1. Compare with graft gonad of Fig. 4.

FIG. 4. Testis of graft recovered from host No. 284. Note absence of central cavity and heterotypic prophases, and the uniform distribution of the germ cells. Compare with the ovary of the host in which this testis developed (Fig. 3). The slender stalk attaching the testis to the mesonephros of the graft is not included in this section.

FIG. 5. Testis of host No. 244, *Amblystoma jeffersonianum*, autopsied 61 days after implantation of graft at stage 31. This gonad exhibits the scattered arrangement of germ cells and the absence of a central cavity noted in the testes of Figs. 2 and 4. Compare with graft gonad shown in Fig. 6.

FIG. 6. Ovary of graft recovered from host No. 244. Although the central cavity is not yet developed, the germ cells are peripheral in position and are for the most part in heterotypic prophase. This gonad thus resembles an ovary (see Figs. 1 and 3) rather than the testes of the host from which it was recovered (see Fig. 5).

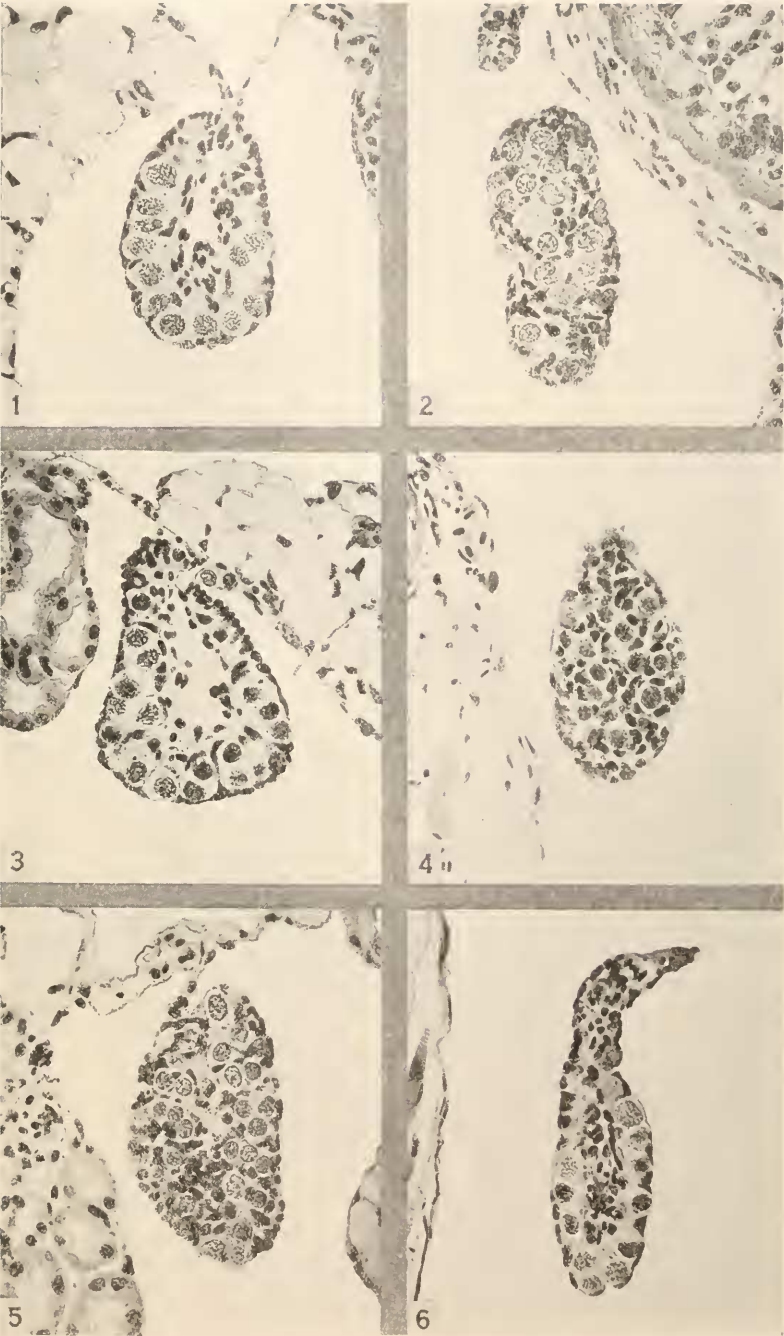


PLATE II. EXPLANATION OF FIGURES.

All figures on this plate are photomicrographs. The magnification is 121 \times except for Figs. 8 and 9, in which it is 162 \times .

FIG. 7. Testis of host No. 207, *Amblystoma maculatum*, killed 61 days after implantation of graft at stage 25.

FIG. 8. Left ovary of donor No. 207, *Amblystoma jeffersonianum*, killed 78 days after furnishing graft (at stage 29) for implantation in host No. 207. Due to the age at which this animal was killed, the ovary is advanced in development as compared with those of Figs. 1 and 3.

FIG. 9. Gonad of graft recovered from host No. 207. Though retarded in its differentiation, this gonad is apparently an ovary, since its germ cells are peripheral in arrangement, and a large proportion of them are in heterotypic prophase stages. Compare with Figs. 7 and 8.

FIG. 10. Ovary of host No. 190, *Amblystoma jeffersonianum*, killed 64 days after implantation of graft at stage 33.

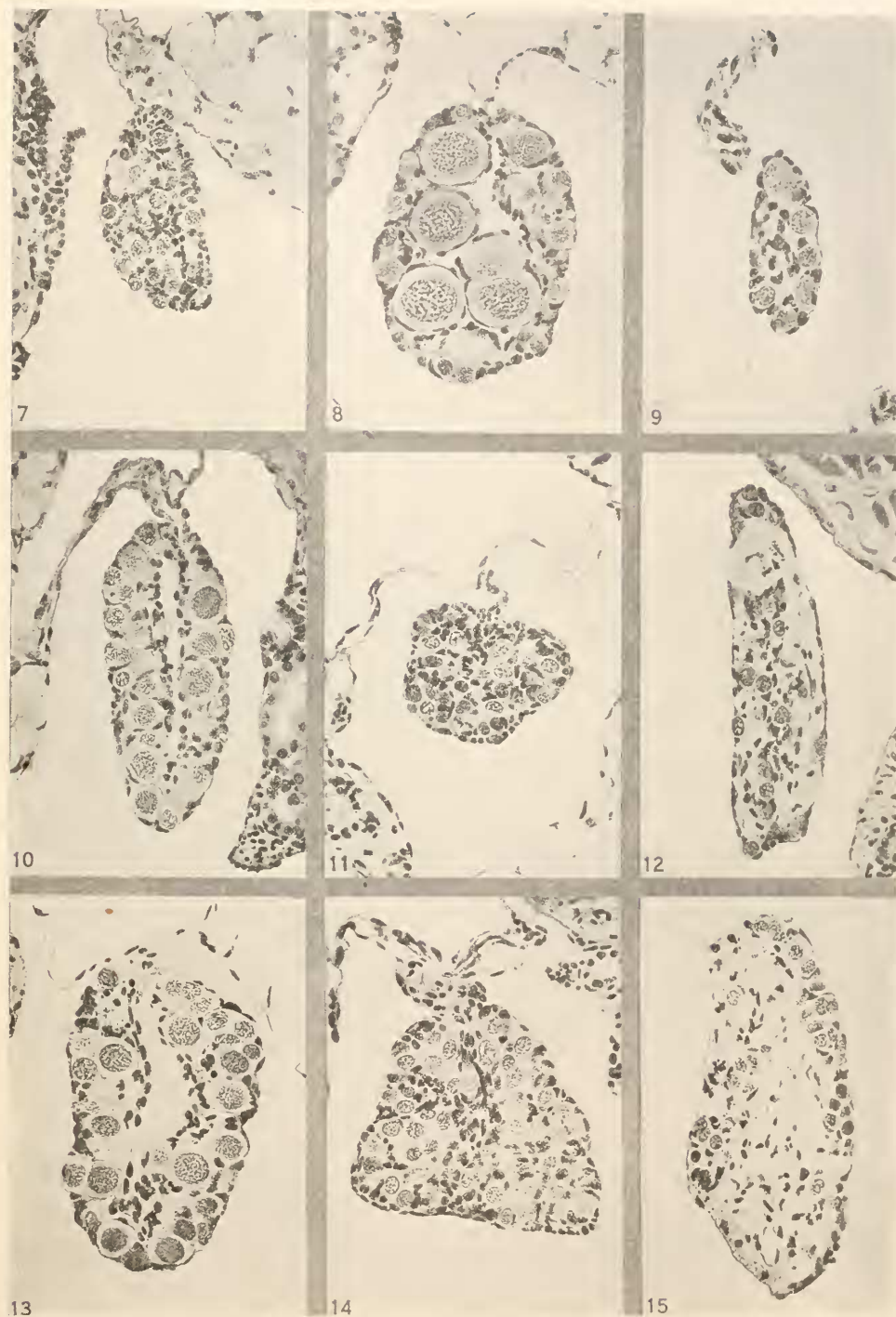
FIG. 11. Left testis of donor No. 190, *Amblystoma jeffersonianum*, killed 64 days after furnishing graft for implantation in host No. 190.

FIG. 12. Gonad of graft recovered from host No. 190. Though atypical in structure, due to the presence of mucous connective tissue, this gonad is apparently a testis. No central cavity is present, the germ cells are scattered, and but very few of them are in heterotypic prophase. Compare with gonad of donor (Fig. 11).

FIG. 13. Ovary of host No. 188, *Amblystoma jeffersonianum*, killed 64 days after implantation of graft at stage 33.

FIG. 14. Left testis of donor No. 188, *Amblystoma jeffersonianum*, killed 64 days after furnishing graft for implantation in host No. 188.

FIG. 15. Gonad of graft recovered from host No. 188. It lacks a central cavity, but has its germ cells predominantly peripheral in position, and frequently in groups or "nests" as in the ovary. Although many of its germ cells are in heterotypic prophase, this is true also of the testis of the donor. This gonad is possibly a testis modified by reason of its development in a graft in a female host.



ON THE PROPERTIES OF THE GONADS AS CONTROLLERS OF SOMATIC AND PSYCHICAL CHARACTERISTICS.

XI. HORMONE PRODUCTION IN THE NORMAL TESTES, CRYPTORCHID TESTES AND NON-LIVING TESTIS GRAFTS AS INDICATED BY THE SPERMATOZOÖN MOTILITY TEST.¹

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I. INTRODUCTION.

Advances in the study of the internal secretions are very often largely dependent upon the development of successful indicators for the substances concerned. Since the studies of Brown-Sequard, innumerable attempts have been made to increase our knowledge of the internal secretions of the sex glands, and indeed vast stores of information have been accumulated through these investigations. The chief difficulty in many of these attempts and especially in attempts to obtain the hormone principle in extractions, has been the lack of an applicable indicator of the substances sought for isolation.

A tremendous step forward in the study of the female hormone was the demonstration of the details of the œstrous cycle as indicated by vaginal smears first by Stockard and Papanicolau ('17) in the guinea pig and later by Long and Evans ('22) in the rat. By the vaginal smear method, one is enabled to determine the presence or absence of substances concerned with the regulation of the œstrous cycle. With such a useful indicator, the advances made in the study of the internal secretions of the ovary have been indeed marked.

On the male side, however, the situation has been a less happy one from the standpoint of real advancement. In some species

¹ This investigation has been aided by a grant from the Committee for research in problems of sex of the National Research Council; grant administered by Prof. F. R. Lillie.

of the bird, notably certain breeds of the domestic fowl, the male feather pattern, behavior, and head furnishings have afforded a good criterion of testicular presence and activity though many details were insufficiently known until of late to make conditions as well understood as was needed. The extensive work of Domm ('27) on the brown leghorn breed has given a much greater appreciation of the many pitfalls that present themselves in this field (for a review of the extensive literature on this subject, see Domm's paper).

When we approach the study of the internal secretions of the gonads in the male mammal, however, a careful analysis will show the marked absence of useful criteria to indicate the activity of the testis hormone, operating over limited periods of time. It is true that there is the sex impulse, supposedly entirely under the control of the internal secretions of the testicles, but many things lead us to believe this supposition to be erroneous. A castrated male theoretically should lose its attraction for the female, but I have repeatedly utilized guinea pigs castrated at 30 days of age as testers for the period of female acceptance for some months after castration. Stone ('27) has recently reported that young male rats castrated at the age of three months will continue to copulate with females for periods of four, five and even eight months. And it is reported that the eunuch, though castrated early in life, will years afterward have not only an attraction toward the female but experiences a degree of satisfaction in this association.

It is likewise true that the growth of the penis, seminal vesicles (when present), prostate, etc., are to a large extent dependent upon the internal secretions of the testis but not only have the variations in such structures proven so great as to make an assay of a given experimental procedure difficult and often impossible, but also, if castrations are made on adult animals, to be followed by such procedures as testis transplantations, injections or other possible approaches, the question of the condition of these structures as representing a balance between postoperative regression or possible stimulation from the materials or conditions utilized often presents insurmountable barriers.

Such other indicators for testicular internal secretions as individual body weight, body length, fat deposition, hair coat,

and pugnacity as have been utilized by other workers serve often to mislead the investigator due to the lack of specificity of the indicator (for further criticisms of this phase see my papers, '21 and '22).

During the course of a study of the physiology of the scrotum or its heat regulating effects on the generative tissues of the testis (Moore, '24*a*, *b*; '26, '27, and '28; Moore and Quick, '24) a possible, fairly satisfactory indicator for the internal secretions of the testis in the differential survival of the capacity for motility of epididymal spermatozoa was discovered by accident. The same conditions were also discovered by Benoit ('26) a little earlier, in the course of his beautiful work on the histology and cytology of the epididymis. The application of this "spermatozoön-motility" test for the testis hormone has been under investigation in this laboratory for longer than three years. The principle of the test may be expressed in details for the guinea pig.

When both testes of an adult guinea pig are removed from the animal, leaving the inferior portion of the epididymides, containing their millions of spermatozoa, in the normal scrotal position, one finds that the spermatozoa gradually lose their capacity for motility when these are suspended in physiological saline solution. A lessened capacity for motility is evident within a few days after testis removal and seldom can one see any degree of motility in the spermatozoa after a period of twenty-three days following the operation. However, if instead of removing both testes, one is allowed to remain normal, the single, opposite, epididymis will contain spermatozoa that show motility when suspended in saline solution for a period of sixty-five to seventy days (Moore, '28). The difference between the 23 days retention of the capacity for motility when both testes have been removed and that of 65 days when one testis has remained, has been proven to be an expression of the internal secretion of the testis (for further details of this reaction see Moore, '28).

While it is freely admitted that the spermatozoön motility reaction has many limitations we have found it very useful and it will continue to be useful until a better hormone indicator has

been discovered. In the following pages a few items of information with respect to its usefulness will be presented.

II. HORMONE PRODUCTION BY NORMAL TESTES.

Utilizing the spermatozoön motility reaction in the guinea pig as described above, I have attempted to study hormone production in the normal testis to learn more concerning its action upon the life of spermatozoa when it is removed from the animal through castration at varying periods during the possible life of the mature germ cell. It has been indicated, for example, that under the influence of the full hormone compliment of one testis, the spermatozoön life, as shown by its capacity to exhibit motion in physiological saline solution, gradually wanes until after approximately 65 to 70 days it no longer responds to this stimulus. Should we, for example, wish to supply the hormone by testis transplantation or by injection of material supposed to contain it, we should be able, if possible, to test the effect in the shortest time within which the reaction will indicate any effect. What influence, therefore, does hormone supplied by a testis *in situ* exert when it acts for ten, fifteen or twenty days? The following procedure will present the method employed.

Young adult guinea pigs are operated under ether anæsthesia through a low mid-abdominal incision and one testis withdrawn into the field of operation. The testis is carefully separated from the inferior pole of the epididymis (tail portion), the internal spermatic vessels are ligated and the testis, along with the head and body of the epididymis removed. The remaining (inferior) portion of the epididymis, connected with its vas deferens, is then carefully replaced in the scrotum—a necessary precaution (see Heller, '29). Ten or fifteen days later the opposite normal testis is removed entire through a scrotal incision. At selected intervals after the second operation, the animals are sacrificed, the isolated epididymis finely hashed with scissors in a small quantity of physiological saline and examined immediately with the microscope for spermatozoön motility. To properly express gradations in motility * signs have been employed in which the normal movement is expressed by ****; the barest vibratile movement on the part of a few spermatozoa (perhaps 1 in 10,000 will contract weakly with little or no trans-

lation) is rated *. Where no movement can be detected the observation is designated o.

Table I. will serve to illustrate the observations on the motility of spermatozoa obtained from the isolated epididymis of animals whose normal testis was allowed to remain for 10, 15, 20, 30, and 40 days after the epididymis to be tested had been isolated.

TABLE I.

UNILATERAL EPIDIDYMAL ISOLATION; OPPOSITE TESTIS REMOVED SUBSEQUENT TO ISOLATION AS INDICATED IN DIFFERENT GROUPS (GUINEA PIG).

Animal.	Epididymal Isolation.	Killed.	Days after Isolation.	Motility.	Excess Life beyond 23 Days Attributed to Hormone Effect.
(delayed removal 10 days)					
517	1-31	3-1	30	*	7 days
518	1-31	3-3	32	*	9 days
520	1-31	3-5	34	o	
521	1-31	3-5	34	o	
(delayed removal 15 days)					
406	6-10	7-11	31	**	8 days
407	6-10	7-14	34	***	11 days
409	6-10	7-20	40	o	
409	6-10	7-20	40	**	17 days
410	6-1	7-24	44	**	20 days
411	6-11	7-29	48	o	
412	6-11	7-29	48	o	
413	6-11	7-29	48	o	
414	6-11	7-29	48	o	
415	6-11	7-29	48	*	25 days
(delayed removal 20 days)					
441	10-21	11-29	39	*	16 days
442	10-21	11-29	39	*	16 days
443	10-21	12-3	43	*	20 days
444	10-21	12-3	43	*	20 days
445	10-21	12-6	46	o	
446	10-21	12-6	46	o	
(delayed removal 30 days)					
450	10-25	12-8	44	**	21 days
447	10-25	12-17	5	*	30 days
451	10-25	12-20	56	*	33 days
452	10-25	12-24	60	o	
(delayed removal 40 days)					
469	12-3	1-25	53	*	30 days
472	12-3	1-25	53	o	
470	12-3	1-27	55	o	
473	12-3	1-27	55	*	32 days
474	12-3	1-31	59	o	

To understand the observations recorded it must be remembered that when an epididymis is isolated from its testis and both testes are removed from the animal, the spermatozoa contained within an epididymis retain their capacity to show motility for a period of 23 days; this we may call the basic life period and realize that they will live for this period without any hormone being produced by the testicle.

From the table it can be seen that under "delayed removal 10 days" the spermatozoa were observed to show motility for 32 days or nine days longer than expected, had both testes been removed at the time of epididymal isolation. We see, therefore, that the hormone supplied by the normal testicle for a period of ten days before its removal, actually extended the life of the spermatozoön nine days. Similarly, hormone supplied for fifteen days extended the retention of the capacity for motility for a similar length of time (actually slightly longer since in animal 415 a few sperm were seen to move slightly on the 48th day after isolation or an increase of 25 days above the basic 23 days expected). When the normal testis was allowed to remain 20 days after epididymal isolation, motile capacity was increased 20 days beyond what it would have been had both testes been removed at the first operation. Hormone supplied by the normal testis for 30 days permitted retention of spermatozoön motility up to 56 days or 33 days longer than the natural life without hormone being supplied. A hormone supply from the normal testis for forty days increased the sperm life by little more than thirty days. But it must be remembered that as we add to the length of time after operation we gradually approach the natural limits of spermatozoön life even with a full complement of hormone; this limit is 65 to 70 days. We could not therefore expect the relative progressive effectiveness to continue much beyond a 40-day normal testis retention because of the approach to the maximum period of persistence of sperm under a continuous hormone influence. In an earlier paper I have emphasized that even utilizing the greatest possible care in the selection of standard animals for operation and in doing the operation itself, there is an individual animal variability that cannot be eliminated; at best we can only make an approach toward quantitative relationships.

Utilizing the basic 23 days as the approximate maximum of retention of the capacity for motility on the part of spermatozoa when no hormone is being supplied (and in scores of observations I have never observed motility for periods above 23 days) we see that one can actually detect the influence of the testis hormone when it is supplied for only ten days. Due to the individual animal variability, I would consider attempts to read reactions more finely as decidedly unprofitable. In fact, to err on the safe side, I have arbitrarily chosen to regard any tested substance or condition involving gonads as negative unless the capacity for motility is retained for thirty days or longer.

It is of interest to examine the data of the above table with reference to what they may tell us of hormone production and storage. When a ten day hormone supply by the normal testis shows an effect of ten days in the reaction, and likewise when spermatozoön motility is extended 15, 20, and 30 days beyond the basic expectations in conditions wherein the testis was present for 15, 20 and 30 days after epididymal isolation, one must conclude, I believe—(1) that hormone secretion is a continuous process and (2) that the hormone is not stored within the body. When hormone is supplied by the normal testis, for 10 days, the reaction indicates an effect for the same length of time.

III. HORMONE PRODUCTION IN CRYPTORCHID TESTES.

It has long been known that man or the domestic mammals may experience a failure of testicular descent into the scrotum. Such animals, although always sterile, nevertheless possess their full compliment of secondary sex characters; they are spoken of as Cryptorchid individuals. The undescended testes of such animals have long been known to lack a germinal epithelium; the gametogenetic function of the testis is deficient but its internal secretory effects are not visibly diminished. It is now known that a normal testis removed from the scrotum and confined within the abdomen very rapidly loses its germinal epithelium and assumes within a month or two, almost identical characteristics to those testes that have never descended. The cause of the degeneration of testes confined within the abdomen has been found to be the warmer environment of the abdomen

and the function of the scrotum has thus been seen to be that of a local thermoregulator (for details of this work see Moore, 1924*a*, 1924*b*, and 1926; Moore and Quick, '24).

Regarding the amount of hormone produced by such a cryptorchid testis, occurring normally or artificially made, little is known. It could be assumed perhaps that a smaller quantity of hormone might be required to produce or to maintain the secondary sex characteristics than would be required to maintain completely normal male conditions. Lipschutz and his co-workers have maintained that in the rabbit 1 per cent. of the normal amount of testicular tissue is sufficient to maintain all the secondary sex characters ('22). On the other hand, it has been assumed by some investigators that any condition leading to an "Apparent increase in interstitial cells" whether by testis transplantation, X-rays, vasoligation (this latter is the basis of the contentions underlying the ideas of the Steinach rejuvenation hypothesis) or any other means, presages an increased production of hormone. The argument proceeds from the assumption—First, that the apparent compensatory hypertrophy of interstitial cells is real (see discussion Moore, '24*a*; Bascom, '25); and second, that the hormone is produced exclusively by the Leydig cells. Some authors have gone so far as to speak of castrated males, bearing testis grafts containing appreciable amounts of interstitial tissue, as "supermales," an implication that I consider without any basis of fact.

In order to gain any new information possible regarding the quantitative aspects of hormone production by cryptorchid testes, I have utilized the guinea pig in the following manner: Young adult guinea pigs have been operated so that one testicle was removed from the scrotum into the abdomen and the inguinal canal closed to prevent scrotal redescend. Four months, and five months, later a second operation was made to isolate the normal epididymis from, and to remove, the normal testicle. We thus have an isolated epididymis with its spermatozoön content to use as a test for the hormone produced by the opposite degenerate four or five months experimental cryptorchid testis.

Table II. is a record of observations made on eleven animals in which one testis was confined to the abdomen for four months

and upon six animals where hormone supply came from a testis confined in the abdomen for five months.

TABLE II.

UNILATERAL CRYPTORCHIDISM FOUR MONTHS; NORMAL TESTIS REMOVED FROM EPIDIDYMISS, AND EFFECT OF FOUR MONTHS CRYPTORCHID TESTIS ON SPERMATOZOÖN MOTILITY DETERMINED.

Animal.	Isolation of Normal Epididymis.	Killed.	Days since Isolation.	Motility.	Wt. of Cryptorchid Testes.*
393	Oct. 13	Nov. 25	43	***	Not recorded
394	Oct. 13	Dec. 5	53	**	0.26 gms.
402	Oct. 17	Dec. 8	52	***	0.424 "
396	Oct. 13	Dec. 12	60	*	0.21 "
397	Oct. 13	Dec. 12	60	o	0.175 "
398	Oct. 13	Dec. 17	65	*	0.158 "
400	Oct. 13	Dec. 17	65	o	0.130 "
403	Oct. 17	Dec. 21	65	*	Not recorded
404	Oct. 17	Dec. 26	70	*	0.170 gms.
416	Oct. 17	Dec. 31	75	*	0.255 "
417	Oct. 17	Jan. 7	82	o	0.205 "

UNILATERAL CRYPTORCHID FIVE MONTHS; SUBSEQUENT TREATMENT SIMILAR TO ABOVE.

475	Apr. 28	June 26	59	**	0.153 gms.
476	Apr. 28	June 30	63	*	0.13 "
477	Apr. 28	July 6	69	o	Not recorded
478	Apr. 28	July 6	69	*	Not recorded
479	Apr. 30	July 12	73	o	Not recorded
481	Apr. 30	July 12	73	*	0.095 gms.

* Testis weight, without epididymis or fat body.

It will be seen from Table II. that spermatozoa within the isolated epididymis have been observed to retain their capacity to show movement on proper stimulation for seventy to seventy-five days. Since the normal testicle, actively carrying on its spermatogenetic activity supplies only sufficient hormone to permit the sperm to live for the same length of time, we must conclude that the hormone producing capacity of a non-gameto-genetic, degenerative, or cryptorchid testicle as measured by the spermatozoön motility test is the equivalent of the normal testis.

Figure 1 is introduced to show the histological character of the testicle after abdominal confinement (experimental cryp-

torchidism) for a period of seven months; the microphotograph shows a portion of a section from the testicle of animal No. 481. This testicle had been confined within the abdomen for a period of five months, when the epididymis of the opposite testicle was isolated for the subsequent spermatozoön-motility test and the normal testicle removed from the animal. Reference to Table II.

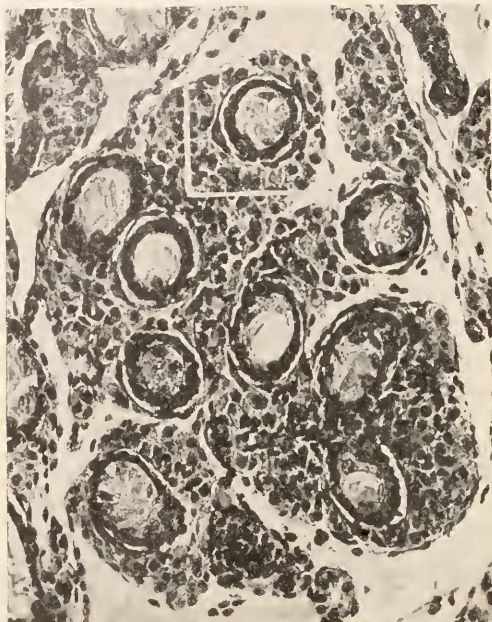


FIG. 1. Photomicrograph of portion of 7 months cryptorchid testicle (animal no. 481) showing shrunken seminiferous tubules separated by interstitial tissue.

will serve to recall that the test epididymis contained a few living spermatozoa 73 days after epididymal isolation and these few exhibited very weak motility on suspension in saline solution. When the animal was sacrificed on the 73d day after epididymal isolation the testicle had been confined in the abdomen for a period slightly longer than seven months. The weight of the organ, after removal of its attached epididymis, was 0.095 grams. Since the average weight of eight normal testicles, without the epididymis, removed from similar sized animals and at the same time of the year, was 1.7 grams (1.34 minimum wt.—

2.06 maximum) it will be appreciated that the weight of this cryptorchid testis represents 2.8 per cent. of the total testicular weight of the normal animal. Had the epididymides been included in this weight the percentage of the normal testicular weight represented by this cryptorchid testis would have been considerably less; the spermatozoön and secretion mass within the normal epididymis being very much greater than the slight fluid content of the cryptorchid epididymis. It can be concluded, therefore, that the cryptorchid testis representing 2.8 per cent. of the normal testicular mass was producing sufficient hormone to maintain the life of spermatozoa in the isolated epididymis for the same period as would the hormone produced by two normal testes.

Figure 1 shows that the seminiferous tubules of the seven month cryptorchid testis were very much reduced in caliber and consisted of a basement membrane, somewhat thickened, and a few Sertoli nuclei and reticulum; the tubules were rather widely separated by interstitial tissue. Fig. 2 is a drawing of the

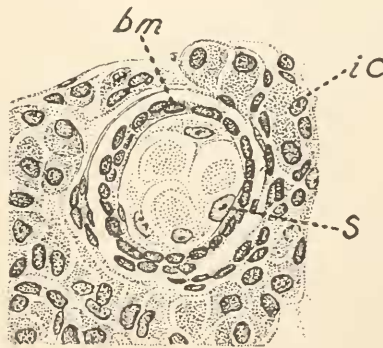


FIG. 2. Drawing of tubule marked off by white lines in Fig. 1. *bm*, basement membrane; *ic*, interstitial cell; *S*, Sertoli nucleus.

tubule marked off by white lines at the upper part of Fig. 1. The thickened basement membrane is more clearly shown and the character of the contents of the tubule indicates an absence of any germ cells; the nuclei that are visible are believed to be Sertoli nuclei. Careful microscopic study has failed to bring to my attention any cell that appeared different from those represented in this figure and it is for that reason that I believe no

germinal cells were present in this testis, at the time of its removal.

IV. HORMONE PRODUCTION BY TESTIS GRAFTS.

The question of the function of testis transplants must of necessity be considered under at least two categories: (1) The function of grafts which have become successfully incorporated into the host organism and remain as living masses of testicular tissue, and (2) the function of such masses of testis tissue transplanted into various parts of the host organism, which by reason of host resistance to the transplant or because of too great a mass of tissue for vascularization, dies and is resorbed or sloughed out of the incorporation bed often with pronounced suppuration. It is known that living testis grafts can be obtained and that they will function. This question, along with the presentation of personal observations, has been reviewed by me at some length (Moore, '26). The question of the function of testis tissue transplanted into a host organism under conditions that have been so unfavorable as to prevent its retention and growth has been dealt with most usually by the clinician. For various reasons many cases of transplantation in man of human testis tissue or testicular tissue from another mammal such as the ram, boar, monkey or deer, have been done. The effects reported are so all-embracing that discredit of all effect is engendered (for discussion of this work see Moore, '26). In general it may be repeated that the effects reported have been expressed in terms of the subjective feelings of the patient—whether he may feel better after remaining quietly in bed for a week or longer after the operation; or whether after the suggestions and discussion of the question and the anticipations of the operation and its outcome, he has a greater desire for coitus; or whether the patient feels that he can walk more sprightly or feels that he can climb a stairs two steps at a time instead of the customary pre-operation one step. In short, such evidence is worthless from the scientific point of view.

In order to study by objective means the question of the function of such non-living testis transplants, I have utilized the guinea pig as the experimental animal and the spermatozoön motility test as an indicator of effectiveness. In an earlier paper

(Moore, '28) I included a few observations then at hand and have since given additional attention to the problem. The method employed, in brief, is the bilateral isolation of the epididymides along with removal of the testes from the animal. The two testes removed were replaced immediately in an especially prepared subcutaneous incorporation bed made by tunneling under the skin, with some destruction of skin musculature and a general scarification of the particular region. Each testicle, cut into two parts, was placed in a separate implantation bed, one on each side of the mid-ventral line of incision.

In addition to the subcutaneous transplantation of the animal's own two testes at the time of epididymal isolation, a few cases of multiple transplantations were studied with the idea that perhaps a small amount of hormone might be liberated from the introduced tissue which if introduced more than once would conceivably show a greater effectiveness. Accordingly at the time of bilateral epididymal isolation, two, one-fourth testes, were introduced subcutaneously at the time of the first operation as well as on the 3d and 5th day following. In each animal, therefore, six transplantations were made, the aggregate amount of tissue transplanted being one and one half testes. The observations on four such experiments are recorded in Table IIIB.

Since an arbitrary choice of thirty days after operation for effectiveness to be registered was made, animals were sacrificed close to this period for the study of the spermatozoön content of the isolated epididymides. From section II. it will be seen that motility of spermatozoa at this time would indicate the equivalent of effectiveness of the normal testis present for seven to ten days. Too much uncertainty surrounds the application of this test to make it profitable to attempt readings at an earlier date.

Table III. presents some of the observations recorded.

Among the nineteen animals whose isolated epididymides were studied for spermatozoön movement between the 25th and 36th day after autoplasmic transplantation of two testes, only two animals have shown any movement of spermatozoa and in each case (animals No. 320, No. 456) observed on the 31st day the motility was the weakest possible for a positive reading. Amid

TABLE III.

A. THE EFFECT OF NON-LIVING TESTIS GRAFTS ON SPERMATOZOÖN MOTILITY (GUINEA PIG).

Animal.	Date— Operation.	Date— Killed.	Days after Opera- tion.	Motility.
364	4- 6-27	5-12-27	36	0 many non-mot. sperm
365	4- 6-27	5-12-27	36	0
370	4- 8-27	5-12-27	34	0 " " " "
371	4- 8-27	5-12-27	34	0 " " " "
372	4- 8-27	5-12-27	34	0 " " " "
373	4- 8-27	5-12-27	34	0 " " " "
320	11-22-26	12-23-26	31	* (1 in 1000 weak mot.)
436	10-15-27	11-15-27	31	* (1 in 1000 weak mot.)
435	10-15-27	11-15-27	31	0
347	3- 8-27	4- 7-27	30	0 many non-mot. sperm
376	4-15-27	5-15-27	30	0 " " " "
377	4-15-27	5-15-27	30	0 " " " "
439	10-20-27	11-19-27	30	0 " " " "
440	10-20-27	11-19-27	30	0 " " " "
437	10-20-27	11-19-27	30	0 " " " "
455	10-26-27	11-25-27	30	0 " " " "
457	10-27-27	11-25-27	29	0 " " " "
458	10-27-27	11-25-27	29	0 " " " "
453	10-25-27	11-19-27	25	0 " " " "

B. MULTIPLE GRAFTS AFTER BILATERAL EPIDIDYMAL ISOLATION.

Animal.	Transplantation Days after Epididymal Isolation.			Killed, Days after Testis Removal.	Motility.
553	1st,	3d,	5th	30	0
554	1st,	3d,	5th	30	0
555	1st,	3d,	5th	30	*
549	1st,	3d,	5th	32	0

the field of millions of spermatozoa, here and there, an individual cell could be seen to show a weak contraction, perhaps a weak vibratile movement every thirty seconds; a rough estimate of 1 in 1000 was made to give a relative notion of the quantity of spermatozoa capable of movement. In contrast to this, eleven animals observed a shorter period of time after operation (25 to 30 days) were all negative; no spermatozoön movement could be seen. One animal (No. 555) receiving six transplantations of one fourth of one testis at three different operations subsequent to testis removal, showed a few spermatozoa capable of weak movement on the 30th day after operation, whereas

two others on the same day and one on the 32d day after operation failed to show any spermatozoa capable of exhibiting movement despite the fact that quantities of normal looking sperm were present.

These results show, therefore, that subcutaneous transplantation of testes provided such a small amount of hormone (if any at all) that its effect was less than the effect of a normal testis remaining *in situ* for seven to ten days after operation.

It is difficult or impossible, as pointed out above, to prevent individual animal variation. Whether the two positive readings on the 31st day are to be explained on this basis of more virile spermatozoa or as an indicator of some hormone effect cannot be stated. But since all operations were done alike, and equivalent masses of tissues transplanted, it would seem as if animals sacrificed earlier (between 25 and 30 days) would have given as strong or a stronger reaction than these three. In any event, should we attribute the results to hormone production and express it as a positive effect of the transplantations, the mildness of the reaction would still be evident. At best it is a questionable indication of hormone production.

The transplanted tissue reactions have been characteristic in all cases. A few days after transplantation the graft site, considerably removed from the line of skin incision, is swollen and decidedly reddened; the elevation caused by the transplanted tissue, at first scarcely visible, becomes approximately the size of a pigeon's egg or larger. It is typically an inflammatory reaction. Ten days after operation the swelling may be almost as large as three days after transplantation and an active pus discharge may be noted. In many cases, the pus spreads toward and escapes through the healing incision but in many cases erosion of the skin over the site of transplantation may provide an escape for the discharge through the new opening. Pus is often seen exuding from such areas up to the termination of the experiment (30 days). In some cases the transplanted mass has so completely sloughed by the termination of the experiment as to be invisible excepting as the site of transplantation may be marked by scar tissue. In other cases small encapsulated masses of pus are present.

It is evident from these observations and considerations that a

mass of testicular tissues undergoing autolysis after transplantation gives little or no evidence of having liberated hormones into the organism.

V. DISCUSSION.

Our three years experience with the spermatozoön-motility reaction as an indicator for the testis hormone have increased our confidence in the test as a dependable objective test for hormone production. Readily admitting that it lacks several desirable qualities to make it entirely adequate for many different approaches to the subject it must still be recognized as a valuable means of studying hormone production.

Relative to our interests here under discussion, we realize for the first time that the hormone produced by mammal testes is not stored within the body of the organism and the internal secretions of this organ are thus brought into line with such other organs producing internal secretions as parathyroids, pituitary, ovary, etc. In the ovarian follicle it has been realized that a temporary storage, perhaps at the site of production, does occur, but that the body does not ordinarily store it up for future release is shown by the failure of recurrence of œstrous in spayed females. Removal of the testis eliminates the hormone source and there is no evidence that any appreciable quantity is retained within the organism. This is especially emphasized when one realizes that a ten day hormone output by a normal testicle expresses itself with an increase of ten days in the length of sperm life (as indicated by their motility); similar additional periods can in general be detected by the reaction.

When the aspermatogenetic or cryptorchid testicle is studied it is indeed interesting to learn that a testicle reduced in weight to 0.095 grams produces as much hormone as two normal testicles carrying on spermatogenetic activity and weighing approximately 3.4 grams. These cryptorchid testes had originally produced sperm, but due to experimental elevation into the abdomen their germinal epithelium had undergone dissolution and removal. Sections show the typical picture of natural cryptorchid testes in that the seminiferous tubule outlines are reduced to small cavities with the characteristic single-celled layer of Sertoli elements; between the tubules the interstitial

cell masses present the typical picture of "apparent interstitial cell hypertrophy." Such anatomical characteristics have been sufficient stimulus for some writers to designate such an animal as a "supermale" but I have never been able to see adequate justification for the introduction of such a term.

The fact that such cryptorchid testes, having a fraction of the weight of the testicular mass in normal male animals, generated the same quantity of hormone, as do two normal testes (judging from the spermatozoön motility test) suggests again speculation as to the actual elements producing the hormone.

It must be admitted that of all possible elements within the testis, the Leydig cells appear to have the weight of evidence in their favor as being the source of origin of the internal secretions. However, since no one has ever satisfactorily eliminated all other elements such as the general connective tissue, but more especially the cells of Sertoli, there remains the same debatable conditions regarding the actual source of origin. When one views the structure of such degenerate testes as these six and seven month cryptorchid testes (cryptorchid four and five months before epididymal isolation and two months of the experiment) and realizes that each is functioning in producing a full hormone quotient (again judging by the test employed) one is certainly inclined to favor the Leydig cells as the source of origin and to minimize the apparently inactive cells of Sertoli. The question however is not yet settled despite the suggestiveness associated with the Leydig cells.

The chief interest connected with the transplanted testis materials was to see if the spermatozoön motility test would reveal the liberation of any hormone during the process of autolysis of the tissue. Since certain writers had reported such imaginary effects from testis transplantation, it was thought possible that during the breakdown of the incorporated tissues some action might be detected. The results of my investigations, however, have failed to show the liberation of sufficient hormone to be detectable. Despite the capability of the test to reveal hormone action for a period of ten days by a normal testicle it becomes evident that should any hormone effect be derived from transplantation of two entire guinea pig testicles, its effect must be less than that of the normal ten-day production period.

As a further defense of the capability of the spermatozoön motility test to indicate the presence of substances produced by the testicle I may mention that subcutaneous injections of lipoid extracts of the bull testicle, prepared by McGee, and injected by me into guinea pigs whose testes had been removed from their epididymides, resulted in prolonging the life of epididymal sperm to the 54th day after operation (Moore and McGee, '28). Therefore, had the transplanted testis masses been liberating hormone into the host organism, we should have been able to detect it by the test under discussion. Such a result certainly lends no credence to the idea expressed by others that similar non-viable testis grafts in man are sufficiently effective to be noticeable for a period of approximately two years, or again that such transplantations are able to aid in the cure of asthma, tuberculosis, myopia, or the host of other debilities attributed to its effect.

The evidence is very direct that as soon as the hormone producing tissues are removed from the organism the lack of the substance produced begins to be detectable in a very short time. No storage within the body for future utilization is evident.

SUMMARY AND CONCLUSION.

The spermatozoön motility test has been found capable of detecting the production of testis hormone for a period as short as ten days.

The hormone produced by the testicles is not stored within the animal body.

An experimental cryptorchid testicle of five months duration, having a weight of approximately one-tenth of a gram, produces as much hormone (indicated by the spermatozoön-motility test) as do two normal testicles at the height of their spermatogenetic activity. The experimental cryptorchid testis is by weight approximately 2.8 to 3.5 per cent. that of the normal testicular mass.

Autoplastic subcutaneous transplantation of two testes results in the liberation of hormone in such small amounts (if at all) as to be scarcely detectable: if any hormone is liberated by these non-viable testis transplants, the effect upon the animal is no greater than the effect of a ten day normal hormone production. So far as present means will indicate, there is no storage or cumulative effect of the hormone.

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INITIATION OF DEVELOPMENT IN *ARBACIA*. V
THE EFFECT OF SLOWLY EVAPORATING SEA-WATER
AND ITS SIGNIFICANCE FOR THE THEORY
OF AUTO-PARTHENOGENESIS.*

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In 1901 Hunter published results of experiments which showed that uninseminated eggs of *Arbacia* exposed to sea-water concentrated by evaporation develop on return to normal sea-water. The present writer has been able to confirm these results though his method differs from Hunter's. The sole reason for reporting the findings here presented is that they lead to some interpretations of significance for Lillie's fertilizin theory of fertilization. The work was done during several summers spent at the Marine Biological Laboratory, Woods Hole, Mass.

THE EXPERIMENTS.

Normal uninseminated eggs of *Arbacia*—free of perivisceral fluid, of high fertilizin content, and capable of giving one hundred per cent. fertilization and cleavage—show a small per cent. of cleavage and of abnormal blastulæ that do not rise to the surface, if after having lain in a small volume of normal sea-water for one or more hours, they are removed to a larger volume of normal sea-water. Two conditions are important for this method of initiating development in the egg of *Arbacia*. First, it is best to use fairly dense egg suspensions. The less dense suspensions prolong the time of exposure necessary to initiate development. Secondly, *it is indispensable for the experiment that the dish containing the eggs be left uncovered to insure evaporation*. A concentration of 1 cc. of "dry" eggs plus 99 cc. of normal sea-water was the least dense suspension successfully used. In some cases it was necessary to leave such a suspension uncovered for twenty-four hours before transfer to the larger volume of sea-water; but it was clear here that evaporation was

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responsible since suspensions of this kind always gave better results when placed in larger dishes with greater surface for evaporation. And if, moreover, a 1 cc. suspension of eggs in 100 cc. of sea-water be poured on a glass plate thereby insuring greater evaporation, the results were indeed striking. However, I am here interested mainly in the results obtained with smaller volumes of eggs and of sea-water.

In all the experiments it was first ascertained that the eggs to be used were in optimum fertilizable condition by trial inseminations for the estimation of their capacity to separate normal membranes. First, the eggs were carefully collected uncontaminated by perivisceral fluid, washed in four changes of 200 cc. of sea-water, and allowed to settle. The supernatant sea-water was decanted and a highly concentrated bulk of eggs thus obtained. For each experiment the eggs were from one female. These were divided into two equal lots whenever their bulk permitted; one lot was placed in an open dish, the other in a glass vial closed with a ground glass stopper. At intervals of 30 minutes a drop of eggs from each lot was removed to 250 cc. of normal sea-water and their development observed. The per cent. of cleavage was as carefully counted as possible, though the count is often made difficult because of the number of cytolized eggs. With further development complete cytolysis of eggs makes the counting of "swimmers" more difficult and of doubtful value since these counts cannot take into consideration eggs that have disappeared through complete disintegration. Moreover, many of the eggs exposed to evaporation develop with their blastomeres separated because the eggs do not possess membranes. In such cases, two "micro-blastulæ" counted may have developed from one egg or each from a different egg, its fellow mass of blastomeres having disappeared completely through disintegration. I therefore early abandoned attempts at making accurate counts and merely noted the presence or absence of "swimmers."

I wish to emphasize that in not one single experiment did I ever find an egg in the stoppered vial that showed the least sign of development. That these eggs were not impaired I determined by inseminating them—in normal sea-water in the case

of highly concentrated suspensions, or in the vials in the case of the less concentrated suspensions.

One other point before we consider the experiments in detail. The reader appreciates the fact that the rate of evaporation varied from day to day. I made no attempt to control this variation. It is also obvious that the rate of evaporation depends upon the volume of solution employed—smaller volumes evaporating more rapidly than larger. Finally, the vessels used make a difference; in my experiments I used either shallow dishes, with a large surface for evaporation, or for the greatest volumes of solutions employed glass plates, 30 x 30 cm. For volumes up to 4 cc. Syracuse watch glasses served admirably.

There now follow a summary (Table I.) of the first type of experiment and a brief comment for the purpose of elucidation.

TABLE I.

THE EFFECT OF SLOWLY EVAPORATING SEA-WATER ON THE UNINSEMINATED EGGS OF *Arbacia* AS SHOWN BY THE PER CENT. OF THEIR DEVELOPMENT ON RETURN TO NORMAL SEA-WATER. EXPERIMENTS ON EGGS OF 45 FEMALES

No.	Bulk of Con- centrated Eggs (in cc.).	Volume of Sea-water (in cc.).	Per Cent. of Cleavage.					Per Cent. of "Swimmers."	
			Exp. No.	1	2	3	4	5	Exp. No. 1.
1	0.1	0.9		8	11	7	14	20	9
2	0.5	0.5		7	0	11	14	3	5
3	0.6	1.2		6	12	4	9	0	4
4	1	1		0	6	9	7	2	0
5	1	1		10	7	8	4	14	13
6	1.5	3		21	17	23	27	18	23
7	2	2		0	3	4	0	5	0
8	2.5	5		13	6	18	21	19	15
9	3	3		14	24	5	0	6	10

Eggs from the same females in stoppered vials: No cleavage, no "swimmers." Same volumes of eggs and sea water in each case except as follows: No. 7, 0.5 cc. of eggs + 0.5 cc. sea-water; No. 8, 0.5 cc. of eggs + 0.5 cc. of sea-water; No. 9, 0.5 cc. of eggs + 1 cc. of sea-water.

The data given in Table I. are for eggs exposed to slowly evaporating sea-water for two hours. This one length of exposure is arbitrarily taken for the purpose of simplicity, instead of presenting the results of each 30-minute exposure. In some



instances the per cent. of development was greater after a longer or a shorter exposure; the results of the two-hour exposure is very nearly the average of all exposures made. Though the per cent. of development in no experiment is high, yet it shows that the evaporating sea-water does initiate development. I do not regard this as an efficient method for experimental parthenogenesis: it has been very suggestive, however, for other lines of my work.

Eggs in sea-water protected against evaporation never show indication that development is initiated. This statement is certainly superfluous for suspensions of uniseminated eggs of *Arbacia* that are ordinarily employed as controls, as all workers know. Of the more dense egg suspensions it might be that lack of oxygen or CO_2 concentration makes initiation of development impossible. The fact that such eggs from such suspension fertilize on return to larger volumes of sea-water does not meet this possible objection. However, I might repeat that some suspensions made of 1 cc. of eggs plus 99 cc. of sea-water exposed to slowly evaporating sea-water showed initiation of development whereas similar suspensions in stoppered vials never did.

Eggs that show initiation of development as the result of exposure to evaporating sea-water never separate membranes, their cleavage is irregular, and the blastomeres tend to fall apart. Many eggs do not cleave and of these some reach the monaster stage with rhythmical dissolution and re-formation of the nucleus. All uncleaved eggs on insemination separate membranes, cleave, and reach the pluteus stage.

The abnormal swimming forms developing from these eggs subjected to treatment with slowly evaporating sea-water never swim at the surface, but merely rotate on the bottom of the dishes; among them are micro- and mega-"blastulae"—i.e., swimming forms developed from blastomeres that have fallen apart and those developed from two or more cleaving eggs. It is this fact that makes difficult the counting of swimmers; hence, the reader will note that except for the first experiment (Table I.) and for one experiment described below (Table II.), I give no counts, but simply note with a + or o sign their presence or absence.

I interpret these experiments to mean that these eggs in

evaporating sea-water are by such evaporation exposed to hypertonic sea-water. It is the hypertonicity that is responsible for the initiation of development and not the mere crowding of the eggs since equivalent volumes of eggs from the same females and of sea-water protected against evaporation do not give any evidence of initiation of development after transfer to larger volumes of sea-water. These eggs as noted above had been thoroughly washed before exposure to evaporation; they would nevertheless continue to produce fertilizin—but so would the eggs protected against evaporation. If fertilizin production, therefore, were responsible for the initiation of development we might expect that at least the highly concentrated eggs in stoppered vials would show some signs of cleavage and farther development. And, what is more, the use of "egg water" instead of normal sea-water does not increase the per cent. or improve the development. Table II. gives the results of a typical experiment on eggs exposed to evaporating "egg water." Drops of eggs from both the uncovered and the stoppered lots were returned at half hour intervals to 200 cc. of normal sea-water. The percentages given are those of eggs having had a two-hour exposure to the evaporating "egg water." This experiment was made five times.

TABLE II.

THE EFFECT OF SLOWLY EVAPORATING EGG WATER ON THE UNINSEMINATED EGGS OF *Arbacia* AS SHOWN BY THE PER CENT. OF THEIR DEVELOPMENT ON RETURN TO NORMAL SEA-WATER. EXPERIMENTS ON THE EGGS OF 9 FEMALES.

No.	Bulk of Concentrated Eggs (in cc.).	Volume of Egg Water (in cc.).	Per Cent. of Cleavage.	Per Cent. of "Swimmers."
1	0.5	1.5	9	7
2	0.5	2.5	13	11
3	1	1	7	0
4	1	1	18	14
5	1	2	11	5
6	2	1	3	5
7	2	2	15	12
8	2	3	12	10
9	2.5	5	9	6

Equivalent volumes of eggs from the same females and of "egg-water," except for No. 9 where 0.5 cc. of eggs and 1 cc. of "egg water" were used, in stoppered vials gave no trace of development after return to normal sea-water.

It would appear from a study of Table II. that there is no advantage in substituting "egg water" for sea-water. As a matter of fact, other experiments with "egg water" gave inferior results. In addition, one gains the impression that exposure to evaporating "egg water" causes more eggs to separate blastomeres, and that there are more micro- and mega-"blastulæ." This I did not properly investigate, *i.e.*, by running experiments on lots of concentrated eggs from the same females, one lot exposed to evaporating sea-water, one to stoppered sea-water, one to evaporating "egg water," and one to stoppered "egg water" counting both the eggs that showed blastomeres falling apart and the micro-"blastulæ." However, some older unpublished observations made independently by Lillie and by the writer may be cited. These showed that "egg water" actually possesses a deleterious effect on development. Thus, I found that if eggs from the same female be divided into two lots, one suspended in sea-water and the other in strong "egg water" before or after insemination, the development of the eggs in "egg water" are markedly inferior to that of the eggs in normal sea-water as measured by the per cent. and normality both of cleavage and of plutei. Lillie also has commented on the adverse effect of "egg water" in other ways on eggs. There is indeed no reason why this should not be true and several reasons why it should. "Egg water" is not simply sea-water charged with fertilizin—it contains products of metabolism of the uninseminated eggs, even though metabolism is at a low level; this would be especially true of eggs highly concentrated in strong "egg water," which perhaps also contains more bacteria than normal sea-water.

Glaser likewise notes that "addition of the extracts ["egg water"] in certain concentrations to normally fertilized eggs, resulted in a retardation of development; normal blastulæ instantly slowed their movements, and underwent a noticeable increase in volume when subjected to the extracts. Similar observations were made on the larvæ of *Arenicola* whose rate of movement was also slowed down, to be followed instantly by an outflow of their yellow pigment and a slight reversible agglutination." Unfortunately, however, Glaser's method of preparing his egg extracts—by removing the eggs directly from the ovaries

into twice their volume of sea-water—is open to objection since he must have carried over some perivisceral fluid. The perivisceral fluid alone may have been responsible for his results.

The following experiment was also made ten times: eggs from one female were placed (1) in sea-water exposed to evaporation, (2) in sea-water in a stoppered vial, (3) in "egg water" exposed to evaporation and (4) in "egg water" in a stoppered vial; at 30 minute intervals drops of eggs were removed from each of the four lots to dishes each containing 200 cc. of normal sea-water. I give now the summary of one long experiment because it shows the results with varying concentration of eggs from one female:

- | | |
|---------|---|
| No. 1. | 10 drops of eggs + 90 drops of uncovered sea-water gave 18 per cent. cleavage, + + "swimmers." |
| No. 2. | 10 drops of eggs + 90 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |
| No. 3. | 10 drops of eggs + 90 drops of uncovered egg water gave 6 per cent. cleavage, + "swimmers." |
| No. 4. | 10 drops of eggs + 90 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |
| No. 5. | 20 drops of eggs + 80 drops of uncovered sea-water gave 27 per cent. cleavage, + + "swimmers." |
| No. 6. | 20 drops of eggs + 80 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |
| No. 7. | 20 drops of eggs + 80 drops of uncovered egg-water gave 8 per cent. cleavage, + "swimmers." |
| No. 8. | 20 drops of eggs + 80 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |
| No. 9. | 30 drops of eggs + 70 drops of uncovered sea-water gave 31 per cent. cleavage, + + "swimmers." |
| No. 10. | 30 drops of eggs + 70 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |
| No. 11. | 30 drops of eggs + 70 drops of uncovered egg water gave 11 per cent. cleavage, + "swimmers." |
| No. 12. | 30 drops of eggs + 70 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |
| No. 13. | 40 drops of eggs + 60 drops of uncovered sea-water gave 21 per cent. cleavage, + + "swimmers." |
| No. 14. | 40 drops of eggs + 60 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |
| No. 15. | 40 drops of eggs + 60 drops of uncovered egg water gave 17 per cent. cleavage, 0 "swimmers." |
| No. 16. | 40 drops of eggs + 60 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers." |

In this experiment because of the rapidity of evaporation on this particular day the eggs were removed to normal sea-water after one hour. The experiment reveals that the effect of evaporating "egg water" is certainly not superior to that of evaporating sea-water in causing initiation of development. It shows also as other experiments cited show that more concentrated suspensions do not yield markedly higher percentages of development than less concentrated ones.

On the whole I think that the evidence which I have submitted indicates that eggs exposed in uncovered dishes develop because of an increasing hypertonicity due to evaporation. Further, the evidence indicates that "egg water" is not necessary for this effect; indeed, "egg water" appears to be less efficacious if not actually more harmful in some small degree than normal sea-water. If this evidence be accepted, Glaser's work on auto-parthenogenesis must be questioned. A brief discussion of Glaser's work and its significance for the fertilizin theory in the light of the work which I herein report now follows.

DISCUSSION.

In 1914 Glaser reported for eggs of *Arbacia* and *Asterias* a type of initiation of development due to exposure to "egg water" for which he chose the name, auto-parthenogenesis. Glaser's procedure was as follows: "Standard secretion ["egg water"] was prepared by adding to a certain number of "dry" ripe ovarian eggs, double their volume of sea-water. At the end of ten minutes, during which the eggs were slightly agitated at intervals, the suspension was centrifuged, and the eggs cast down. After 100 revolutions the supernatant fluid was carefully decanted and set aside for use.

"Ripe eggs were then shaken, usually from the ovaries of a single individual, into a small quantity of fresh sea-water, and to 1 cc. of a concentrated suspension of these was added 1 cc. of the secretion. In this mixture the eggs were allowed to stand 2 hours, when cleavages were usually found in all the dishes."

And further: "Many experiments were tried varying the concentration of the secretion as well as the time of exposure. My records indicate cleavages at higher concentrations as well as lower, and also in less than two hours, but the greatest number

was always obtained when 1 volume of the concentrated egg suspension was exposed for 2 hours to 1 volume of the standard secretion. If at the end of this time the supernatant fluid is poured off and replaced by fresh sea-water, free swimming blastulæ will be found within 24 hours. In one case only did development proceed to the pluteus stage."

As I have stated above, Lillie was never able to repeat this observation made by Glaser. Nor was I until by chance I observed the extent of evaporation that had taken place in two cc. of egg water put in a Syracuse watch glass one hour before. Deliberately repeating this chance observation on eggs suspended in either "egg water" or sea-water through several seasons I have obtained initiation of development in *Arbacia* eggs provided the "egg water" or sea-water be allowed to evaporate. I am therefore constrained to believe that Glaser's auto-parthenogenesis is a hypertonic effect due to evaporation.

Glaser has also reported what he calls an improved method of auto-parthenogenesis. Says Glaser: "Loeb's improved method of artificial parthenogenesis consists in following the treatment with parthenogenetic agents, by an after treatment with hypertonic sea-water, 8 cc. of 2.5 *M* NaCl plus 50 cc. of sea-water. It seemed likely, therefore, that a better yield of larvæ could be secured if eggs, after having been subjected to the action of the secretion for two hours, were afterwards treated with the hypertonic solution for forty minutes. This surmise proved correct." The proof offered is the outline of a typical experiment showing the development in two sets of eggs *both* of which were exposed to hypertonic sea-water after treatment with the egg secretion. There are, it seems to me, two objections to this experiment.

In the first place, in the improved method of artificial parthenogenesis Loeb typically used butyric acid which alone is not capable of causing development of the egg beyond the separation of the vitelline membrane and formation of a monaster around the egg nucleus; according to Glaser, the egg secretion which he used causes development at least to the blastula stage without separation of membranes. Moreover, when one uses butyric acid one must replace the acid sea-water with normal sea-water and allow a certain time to elapse before beginning the treatment

with the hypertonic sea-water; Glaser exposed his eggs to the egg secretion and at once transferred them to the hypertonic sea-water. There is here, therefore, no similarity between the improved method of Loeb and that of Glaser.

Secondly, and this is far more serious, Glaser does not tell us to what extent there is an improvement through the after treatment with the hypertonic sea-water; he gives no information concerning the development of two lots of eggs from the same female, one with and one without hypertonic sea-water after the exposure to the egg sea-water. Obviously, Glaser should have set up an experiment on four lots of eggs from the same female, one an uninseminated control in normal sea-water, one exposed to hypertonic sea-water alone, one to egg water alone, and one to hypertonic solution after a treatment with "egg water."

In the same communication Glaser also described auto-parthenogenesis in eggs of *Asterias*. For this he used either 1 or 2 volumes of maturing *Asterias* eggs plus one of "egg water" and obtained fertilization membranes, cleavage, and "much gastrulation." I would suggest that this result was due in part to CO₂, which in *Asterias* eggs initiates development, and to hypertonicity.

Glaser's "hetero-parthenogenesis" is the effect of *Arbacia* "egg water" on *Asterias* eggs. Here again he used 1 volume of "egg water" (from *Arbacia* eggs) to 1 volume of *Asterias* eggs. The foreign "egg water" gave fertilization membranes and numerous cleavages. I venture the opinion that the initiation of development was due to one, two or a combination of three factors: CO₂, hypertonicity, and the foreign perivisceral fluid which from Glaser's method of procuring the *Arbacia* "egg water" must have been present.

On the basis of my findings and the possibility that these adverse criticisms of Glaser's work be correct, I suggest that auto-parthenogenesis is an initiation of development due to hypertonicity of either "egg water" or sea-water. If this be true Glaser's criticisms of Lillie's fertilizin theory based on his findings are without foundation.

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INTRACELLULAR HYDRION CONCENTRATION STUDIES.

I. THE RELATION OF THE ENVIRONMENT TO THE pH OF PROTOPLASM AND OF ITS INCLUSION BODIES.

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Recent micrurgical investigations (1, 2, 3) on the colorimetric determination of the protoplasmic pH have emphasized the need of studying the relation between the pH of the protoplasm of a living cell and that of its environment. Of the acids and bases which affect the pH of the environment some penetrate living cells while others apparently do not. This has been demonstrated by the change in color of cells stained with indicators. For example, with the use of neutral red it has been shown by previous investigators (4, 5) that living cells are readily permeable to CO_2 and NH_3 but not to HCl nor NaOH . This fact that the color of the intracellular stain can be readily shifted to the acid or the alkaline side suggests that the intraprotoplasmic pH can be changed very easily by environmental conditions, a conclusion which is at variance with experiments which indicate that protoplasm has a marked buffering power. Thus, when solutions of indicators, both in the acid and the alkaline states of their color ranges, are injected into living cells the colors quickly shift to those characteristic of a constant pH (6.9 ± 0.1). This has been found true for such varied types of cells as the ameba (1, 6), marine ova (2, 3), and various tissue cells of the frog and the mammal (6). In addition, there is the significant result that the localized increase in intraprotoplasmic acidity, caused by mechanical injury is almost immediately neutralized as long as no cytolysis results (1, 2, 3, 6).

In view of these facts it was considered advisable to test further the constancy of the intraprotoplasmic pH, to discover

whether this pH can be shifted appreciably without detriment to the cell and to obtain evidence, if any, of localized variations in the intracellular pH.

The purpose of the experiments described in this paper is to determine whether the intraprotoplasmic pH can be shifted by exposure to CO_2 or to NH_3 and whether the reaction to indicators of such intracellular structures as granules and vacuoles are comparable to those of the optically homogeneous protoplasmic matrix.

Before dealing with the actual experiments performed it is necessary to describe the manner in which the protoplasm becomes colored with neutral red and with the other dyes used. When cells are stained with neutral red or certain other basic dyes, the dye accumulates in or on the intracellular granules and vacuoles while the hyaline protoplasmic matrix remains colorless. This occurs not only when cells are stained by immersion in a solution of the dye but also when the dye is injected directly into the cell. In the latter case the color appears at first diffuse but gradually the granules and vacuoles take up more and more of the color until none of it can be detected in the hyaline cytoplasmic matrix. On the other hand the acid dyes used, e.g., brom cresol purple, phenol red and cresol red, do not penetrate from the environment into the cells. When injected, however, they quickly spread through the cytoplasm giving to its hyaline matrix a more or less permanent and diffuse coloration (1, 2, 3, 6).

The fresh water *Amœba dubia* and the unfertilized eggs of the starfish, *Asterias forbesii*, and sanddollar, *Echinarachnius parma*, were used in these experiments. The amœba and the eggs were colored with the dyes either by the immersion method or by the microinjection method. Both methods were also used simultaneously on the same cell. The cells were then immersed in various acid and alkaline solutions and the color changes noted. For a study of the effect of NH_3 and CO_2 the cells were suspended in hanging drops of water from the roof of a special form of moist chamber which was closed except for narrow inlet and outlet tubes (7). The hanging drops were then charged with either CO_2 or with NH_3 by passing the moist gas through the chamber.

1. EFFECT OF ACIDS AND BASES ON AMEBÆ COLORED BY THE INJECTION OF ACID INDICATORS ONLY.

Amebæ were injected with 0.4 per cent solution of brom cresol purple, phenol red and cresol red (8). These indicators were selected because they change color within the pH ranges tested (1, 3). Amebæ, injected with brom cresol purple, are uniformly blue (the alkaline range), with phenol red, a pale orange yellow (approaching the acid range). These findings accord with those already published (6) from which the pH of the freshwater ameba was placed at 6.9 ± 0.1 .

Amebæ, colored by the injection of the above-mentioned dyes, were immersed in solutions of HCl (pH 5.5), NH_4Cl (pH 5.5), CO_2 charged water (pH 5.5), NaHCO_3 (pH 8), NH_4OH (pH 8) and NaOH (pH 8). The acidity of the first three solutions is sufficient to cause the indicators to take on the yellow color of their acid ranges, while the alkalinity of the last three solutions is sufficient to give to brom cresol purple the purple blue, and to phenol red and cresol red the bright red color of their alkaline ranges. It was found that the immersed amebæ all maintained their original colors as long as they remained alive. The color of those which rounded up and died changed to that characteristic for the pH of the environing medium.

These results indicate, either that there is no penetration of the acid or of the alkali from the solutions used, or that the protoplasm is sufficiently buffered to neutralize the acid or the alkali which does penetrate.

2. EFFECT OF ACIDS AND BASES ON CELLS STAINED WITH NEUTRAL RED AND INJECTED WITH ACID INDICATORS.

a. *Amæba dubia*.

Since the permeability of cells to certain acids and bases can be demonstrated by the change in color of neutral red, amebæ were immersed in a solution of neutral red until various intracellular inclusions took on a red color. These amebæ were then injected with solutions of the indicators which color the cytoplasm diffusely. On immersing these doubly colored amebæ into the various acid and alkaline solutions the following results were obtained:

In accordance with the previous experiment it was found that immersion produced no change whatever in the diffuse coloration of the hyaline cytoplasmic matrix. On the other hand, the inclusion bodies which were stained with neutral red quickly became yellow in the solutions containing the NH_3 (NH_4OH and NH_4Cl) and bright red in those containing CO_2 (NaHCO_3 and CO_2 charged water).

These results imply that the pH of the hyaline cytoplasm does not change even when sufficient NH_3 or CO_2 penetrates to change the color of the intracellular inclusions. In other words, the pH of the intracellular inclusions can be shifted readily by the presence of CO_2 or of NH_3 in the environment while that of the protoplasmic matrix remains constant.

b. Unfertilized Eggs of the Sanddollar (Echinarachnius parma) and the Starfish (Asterias forbesii).

The protoplasm of these eggs is uniformly crowded with granules or macrosomes practically all of which ultimately stain a deep rose red with neutral red. The eggs were allowed to remain in sea-water containing neutral red only long enough to stain a small percentage of the granules. The eggs were then washed, transferred to hanging drops of sea-water in the moist chamber and injected with the indicator solutions. In the same chamber were placed, as controls, other hanging drops of sea-water colored with the same indicators. Ammonia gas was then passed through the chamber until the hanging drops became sufficiently saturated with ammonia to change the color of the control drops.

The color of the eggs was noted when the dyes in the control drops had assumed colors indicating a pH more alkaline than 8.4. In every case the color of the granules, stained with neutral red, changed from red (acid) to yellow (alkaline) while the diffuse coloration of the indicators in the hyaloplasm of the eggs persisted in registering the originally recorded pH of 6.8 ± 0.1 (3).

An experiment giving striking color contrasts is one in which three dyes, neutral red, phenol red and cresol red, were used for the purpose of detecting simultaneously the pH changes in the cytoplasm, the cytoplasmic granules, and the sea-water surrounding the eggs. It is to be remembered that neutral red

which stains the granules is red at a pH more acid than 6.8 and yellow at a pH more alkaline than 7.4. Phenol red which colors the hyaloplasm is yellow at a pH more acid than 6.8 and red at a pH more alkaline than 7.4, and cresol red which was used for the environing sea-water is yellow at a pH more acid than 7.8 and red at a pH more alkaline than 8.0. The experiment was the following: Eggs, stained with neutral red, were immersed in a hanging drop of sea-water colored with cresol red and were then injected with phenol red. The result was a striking picture of yellow eggs containing scattered red granules and surrounded by a medium of yellow sea-water. Ammonia gas was then passed through the chamber until the cresol red in the sea-water changed from yellow (acid) to red (alkaline). As soon as this occurred the cytoplasmic granules, stained with the neutral red turned yellow (alkaline) while the hyaloplasm maintained the original yellow (acid) color of the phenol red. The result was now a picture of uniformly yellow eggs standing out against a background of red sea-water. Carbonic acid gas was then passed through the chamber until it displaced the NH_3 in the hanging drops. As a result the original colors returned, viz., the sea-water again became yellow, the cytoplasmic granules turned from yellow to red but the cytoplasm itself remained yellow.

Since the cytoplasm has a pH of 6.8 ± 0.1 (3) which is in the acid range of phenol red the above experiment is not suited for detecting a possible effect of the CO_2 on the cytoplasmic pH. For this purpose it is necessary to use brom cresol purple (yellow at a pH more acid than 6.0 and blue at a pH more alkaline than 6.2) which, upon injection, colors the hyaloplasm blue. These eggs were immersed in a hanging drop of sea-water colored blue with the same dye. The hanging drop was suspended in the hermetic chamber through which moist CO_2 gas was made to stream until the sea-water became charged with CO_2 sufficiently to change its color from blue to yellow. The eggs in the yellow water kept their original blue color.

These experiments indicate that NH_3 and CO_2 , both of which penetrate the protoplasm and affect the pH of the intracellular granules, do not shift the pH of the hyaloplasm as measured by the indicators.

3. EFFECT OF CO_2 AND OF NH_3 ON AMEBÆ WHOSE CYTOPLASM
AND INCLUSION BODIES ARE COLORED
WITH THE SAME INDICATOR.

A possible error in the previous experiments lies in the fact that the coloration of the cytoplasmic inclusions and of the hyaline cytoplasm were not made with the same dye. For example, neutral red, which colors the cellular inclusions, is a basic dye, while the dyes used for producing a diffuse coloration are acidic. It is conceivable that this may be responsible for their difference in reaction to the penetrating CO_2 or NH_3 .

To meet this objection it was found that methyl red could be used. Methyl red has already been used as a vital stain for plant protoplasm (9) and is a pH indicator, being red at a pH more acid than 5.0 and yellow at a pH more alkaline than 5.4. Immersion of amebæ in an aqueous solution of this dye stains the hyaline cytoplasm, its various inclusions and the nucleus an intense yellow. Amebæ colored in this way were placed in a moist chamber in hanging drops of the yellow aqueous solution of methyl red. Moist CO_2 gas was then passed through the chamber until the hanging drops turned from yellow to red. When this occurred it was found that the yellow stained inclusions of the ameba had also become red while the cytoplasm and nucleus remained yellow. Ammonia vapor was now passed through the chamber whereupon the color of the hanging drops and of the intracellular inclusions quickly changed back to yellow.

These experiments with methyl red clearly demonstrate the penetration of CO_2 into the living ameba¹ as registered by the change in color of the intracellular inclusions. The hyaline cytoplasm and the nucleus, however, maintain their original color and give no evidence of a change in pH.

¹ The neutral red method is not very favorable for detecting the penetration of CO_2 into cells since the granules stained with neutral red under normal conditions already have the rose red color characteristic for the acid range of the dye. On the other hand, methyl red under normal conditions stains the intracellular granules the yellow color of its alkaline range. Upon exposure to CO_2 the color of the granules changes to red, which is as decided an evidence for the penetration of the CO_2 as is the neutral red method for the penetration of NH_3 .

4. THE EFFECT OF PENETRATING ACIDS AND BASES ON THE NUCLEAR pH.

The nuclei of immature starfish eggs were used in these experiments. The nuclei of different eggs were colored with cresol red, neutral red and phenol red by the microinjection method after which the eggs were exposed to CO_2 and to NH_3 . In every case the color within the nuclei of living eggs remained constant irrespective of the color changes of the granules in the surrounding cytoplasm. In other words, the nucleus was found to be sufficiently buffered so that the intranuclear pH of 7.6–7.8 (3) remains unchanged. When the egg disintegrates by crushing or tearing, the nucleus undergoes changes (3) and loses all buffering action. The persisting spherical nuclear remnant is then immediately susceptible to acid and alkali changes in its environment.

SUMMARY.

The presence of CO_2 or of NH_3 in the aqueous medium surrounding living cells readily changes the pH of the intracellular inclusions which stain with neutral red but does not change the pH of the protoplasmic matrix nor of the nucleus as long as the cell is alive.

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INTRACELLULAR HYDRION CONCENTRATION STUDIES.

II. THE EFFECT OF INJECTION OF ACIDS AND SALTS ON THE CYTOPLASMIC pH OF *Amæba dubia*.¹

PAUL REZNIKOFF AND HERBERT POLLACK.²

In a previous communication (1) from this laboratory the pH of the cytoplasm of *Amæba dubia* was reported to have a value of 6.9 ± 0.1 . To determine whether any permanent variations in the intracellular pH could be artificially produced, solutions of acids salts and simple salts having toxic actions were injected by the micrurgical technique (2) into amebæ previously colored with indicators.

EXPERIMENTAL.

The ameba and methods used in these experiments were the same as those described previously (2). The hydrion indicators (3) employed were thymol blue, brom phenol blue, brom cresol green, methyl red, chlor phenol red, brom cresol purple, phenol red, and orange III. Of these phenol red was the most extensively used. The advantages of this dye are twofold. It is the least toxic of all the indicators and is the most valuable one in experiments of this type since its useful range covers the normal cytoplasmic pH. The other indicators were used in extreme changes of pH.

In the case of each solution, at least 10 amebæ were used, and for critical concentrations from 25 to 50. Small quartz cover slips were employed in these experiments. They were attached by means of water films to the ordinary long glass cover slips. On the quartz slips were placed amebæ in a drop of their medium, varying in reaction from pH 5.8 to 7.5, a drop of indicator, and

¹ From the Laboratory of Cellular Biology, Department of Anatomy, Cornell University Medical College, New York City, and the Marine Biological Laboratory, Woods Hole, Massachusetts.

² Expenses connected with this investigation were in part defrayed from a grant by the Ella Sachs Plotz Foundation.

a drop of the solution the effect of which was to be tested. The pipettes used were made of pyrex glass and were rinsed several times in distilled water and then in solutions of the substances to be injected. The dyes were injected into the amebæ which were permitted to recover. After recovery the next solutions were introduced.

As a control, the degree of injury caused by the insertion of the pipette was determined. The method employed was to note any change in color of the previously injected dye from the possible formation of acid associated with injury (4). The simple introduction of a pipette into an ameba was found to give no indication of acid production. If the pipette, however, stirred up the cytoplasm so vigorously that the injured area was ultimately discarded, a distinct acidity was produced. When death occurred in the presence of those dyes covering the range, a pH of about 5.5 was indicated, unless the pellicle surrounding the mass was broken in which case the color was rapidly washed out.

Solutions of HCl (pH 2) when introduced into an ameba, which is colored an orange-yellow with previously injected phenol red, cause an immediate and intense yellow coloration of the injected area. If the injected region is not irreparably injured by the acid the pH of the area reverts within a few seconds to that of the normal cytoplasm. Usually, however, the injected portion is injured to such an extent that it is pinched off in a manner previously described (2) after which the yellow color (acid) of the discarded sphere gradually changes to that indicative of the pH of the environment. In time the color entirely washes out. The unaffected remnant of the ameba retains its orange-yellow color.

When CaCl_2 is injected in concentrations stronger than $M/200$ the phenomenon of solidification and pinching off is accompanied by distinct evidence of acid production. If a solution of $M/200$ CaCl_2 is injected into amebæ colored with phenol red, the flash of yellow color indicating acid production rapidly returns to that of the normal pH, provided the injected area is not discarded. If the color does not revert within a few seconds the affected portion is pinched off.

In only three cases out of several hundred did the maintenance of a localized acid reaction persist for as long as a minute after HCl or CaCl_2 had been introduced without subsequent pinching off. To investigate further this condition in which a localized acid reaction is maintained for an appreciable time with subsequent complete recovery solutions of $M/32 \text{ AlCl}_3$ were introduced into amebæ colored with phenol red. Such a concentration of AlCl_3 causes a solidification of the injected portion but this region is not infrequently reincorporated after being almost discarded (5). Of at least 50 cases only one showed a delay of two to three minutes in the return of color from yellow (acid) to the original orange-yellow after the affected area had been reincorporated. In every other test the reversal of color was immediate if reincorporation occurred or, if the area was discarded, its color remained yellow.

The introduction of solutions of MgCl_2 of pH 6.5 in concentrations of $M/30$ and stronger into amebæ previously injected with phenol red causes an immediate shift to yellow, indicating acid production. The cell breaks and the color diffuses out. When an $M/60$ solution of MgCl_2 is injected the yellow color reverts rapidly to that normal for healthy cytoplasm and the ameba recovers.

In order to determine the degree of acid production by the injection of CaCl_2 and MgCl_2 (pH 6.6) amebæ were injected with this salt after having been colored with thymol blue, orange III, methyl red, brom phenol blue and brom cresol green. All these dyes were injected with the exception of methyl red (6). Amebæ were stained with methyl red by immersing them into 5 cc. of distilled water into which a few drops of a 0.4 per cent. aqueous solution of methyl red were placed. With methyl red a distinct red is produced when either CaCl_2 or MgCl_2 is injected into amebæ. With orange III the yellow color persists. This places the reaction of the acidified portion of the cell between pH 4.0 and 4.6. It is difficult to determine a more exact pH value because the color changes with brom phenol blue are not sufficiently distinctive within the critical range. These results show that the acid produced by injection of CaCl_2 or MgCl_2 is more marked than the acid of injury which was found to be about pH 5.5.

When NaCl and KCl (pH 7) are injected into amebæ colored with phenol red, no immediate change in color occurs. If the concentration of these salts is lethal (2) the rounded amebæ gradually take on the color indicative of the pH of the environment. Injection of non-lethal concentrations of these salts results during the quiescent period in a slight shift in color toward that suggestive of the pH of the surrounding medium whether this be acid or alkaline. But as soon as the ameba recovers the color reverts to the normal orange-yellow.

The change in color of the discarded spheres after HCl and CaCl_2 had been injected or of the dead ameba when lethal amounts of NaCl or KCl were introduced is due to the penetration from the environment. It is quite obvious that any uncontrolled changes in the environmental pH would be confusing. Therefore it was necessary to take precautions to obviate this factor. In preliminary experiments, when ordinary cover slips were used the medium increased in alkalinity markedly during its contact with the coverslip. The use of pyrex glass or coverslips coated with balsam or collodion did not prevent this change in hydrogen ion concentration. To maintain a constant pH of the environment a buffer calcium acetate solution ¹ of pH 6 was used. In this amebæ were immersed and the various salts and acids injected. In this case the dead spheres and the amebæ killed with NaCl or KCl took on the color representing the reaction of the surrounding medium, viz., pH 6. With quartz cover slips, which do not affect the pH of solutions coming into contact with them, the medium remained constant and the discarded spheres and dead amebæ assumed the reaction of any environing medium into which they were placed. These results show that the injection of the individual chlorides are ineffective in changing the intracellular pH except when toxic concentrations were used.

DISCUSSION.

In the marine egg the production of acid due to injury is much more easily manifested than in the ameba. As previously shown (4) a localized flash of color indicating acid production is apparent in the starfish egg if the needle is introduced abruptly

¹ We wish to thank Dr. William Perlzweig for the preparation of this buffer solution.

into the interior. This change is not evident in the ameba unless the mechanical trauma is vigorous enough to cause death of the disturbed part. This difference points to a greater susceptibility to injury of the egg or an increased buffering power of the ameba which may in turn be due either to an increase in the amount of buffer present or to a greater ease in diffusion of buffers through the cell. This faster rate of mobilization of buffers in the ameba as compared to the egg is suggested by the constant flow of cytoplasm of the ameba in contrast to the relatively 'quiescent' cytoplasm of the egg.

The production of acid when CaCl_2 or MgCl_2 is introduced into the ameba may be due to the production of insoluble Ca or Mg salts with the liberation of free acid. Aub and Reznikoff (7) have suggested such an explanation for the effect of Pb salts on red blood cells. Ca may also unite with the carbonate and phosphate to form insoluble salts with the production of free acid. This acid formation is evident until some alkali diffuses into the solidified mass and neutralizes the acids present. Such an explanation does not preclude the possibility also of the formation of a Ca or Mg organic compound.

CONCLUSIONS.

1. The cytoplasm of the living *Amæba dubia* shows considerable buffering power to pH changes induced by the injection of salts and buffers.
2. If HCl, injected into the ameba, is immediately buffered by the cytoplasm no toxic effect results. If the quantity injected is too great to be buffered, the affected portion of the cell dies and is discarded.
3. CaCl_2 , MgCl_2 and AlCl_3 , injected into amebæ colored with indicators, give colorimetric evidence of the production of acid greater in amount than can be explained by acid produced by mechanical injury. Unless this color reverts immediately to that indicative of normal cytoplasm, the affected portion is discarded in the case of CaCl_2 and AlCl_3 and the entire cell dies in the case of MgCl_2 .
4. Upon death permeability changes occur so that the dead mass of the ameba quickly assumes the hydrogen ion concentration of the environment.

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INTRACELLULAR HYDRION CONCENTRATION STUDIES.

III. THE BUFFER ACTION OF THE CYTOPLASM OF *Amæba dubia* AND ITS USE IN MEASURING THE pH.

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Recent determinations of intracellular pH have been made by noting the color of indicator dyes injected directly into the protoplasm (1, 2, 3, 4, 5). The recorded value was found by comparing the results of injecting a series of overlapping dyes. The color of the dye, whose range was found to include the pH of the cytoplasm, was compared with known standards projected optically into the field of the microscope.

While attempting to determine the buffer action of the cytoplasm it was found that an indirect method could be used to check the results obtained from the direct color comparisons.

It is known that a drop of a solution at a certain pH added to another buffer solution containing an indicator dye will cause a momentary localized change in the color providing the reactions of the two solutions are different. The closer the pH values of the two solutions are to one another, the less marked will be the color change. When they have the same pH there will be no change in color. It is possible to take advantage of this fact in measuring the intraprotoplasmic pH by injecting a series of solutions of known pH into cells colored by the previous injection of indicator dyes. As will be brought out later, this technique is only approximate but serves to check wide deviations from direct tint comparisons.

It has been shown that $M/4$ solutions of mono-sodium phosphate may be injected with no toxic effect (6), and that the potassium ion has about the same toxicity as the sodium ion on injection (7). Hence the Clark buffer solutions (8) whose KH_2PO_4 concentration is $M/20$ should be non-toxic from the point of view of salt concentrations, and any toxic effect must be due to the buffered hydrogen ion concentration.

Amoeba proteus and *Amoeba dubia* were used in this study since their pH has been determined by direct tint readings.

The amoebæ were injected with brom cresol purple and phenol red which were the indicators whose ranges cover the pH as found by previous work. The colored amoebæ were then injected with the phosphate buffers from pH 5.6–8.0 and observations made on the changes in color.

When buffer solutions of 5.6, 5.8, 6.0 were injected into amoebæ colored with brom cresol purple, a temporary but distinct yellow flash was produced. Those above 6.2 produced no color change with this indicator which is already blue in the cell. Buffer solutions of pH 6.2 and 6.4, when injected into amoebæ colored orange yellow with phenol red, gave temporary yellow flashes. With the same indicator, solutions having a pH of 6.6, 6.8 or 7.0 showed no color change. Those whose pH was 7.2 and above showed reddish flashes in the orange yellow colored cytoplasm.

This shows that the pH of the amoeba is not less than 6.6 and not greater than 7.2. This is in accord with the results obtained in this laboratory in previous investigations and not with those obtained by the Needhams (2). They also used the microinjection technique with direct color comparison for reading the pH values. Their value for the cytoplasmic pH of the amoebæ was 7.6, as was Pantin's, who used the neutral red vital staining technique (9).

As for the Needhams' results it must be remembered that they were using a European species and also that they report the amoebæ died within five minutes after injection. In the investigation reported in this paper the amoebæ were allowed to recover fully after the injection before treatment with the buffer solutions. With a proper injection of phenol red and brom cresol purple, amoebæ can be kept alive and apparently normal for at least two days (4). Frequent checks on the color by direct comparison with standard buffers showed no change during that time. The amoebæ colored with phenol red maintained the same orange yellow tint (pH 6.9 ± 0.1) as long as they were kept under observation. On the other hand moribund and dead amoebæ take on the pH of the environment which is usually alkaline when the ordinary glass coverslips are employed without proper

precautions (5). As for the value obtained by Pantin (8) the inefficacy of neutral red staining has been shown (3, 10).

The interesting fact is that regardless of the pH value of the buffer solution injected the return of color of the indicator present to its usual one is quite rapid and constant. If, however, sufficient buffer was put in to change the pH of the cell, the cell died. These facts emphasize two important points relating to intracellular hydrogen ion concentration. One, that the cytoplasm has a considerable buffering power, and two, that when the pH of the cytoplasm is changed, the cell dies.

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THE EFFECTS OF CHANGES IN MEDIUM DURING
DIFFERENT PERIODS IN THE LIFE HISTORY
OF *UROLEPTUS MOBILIS* AND
OTHER PROTOZOA.

LOUISE H. GREGORY.

3. THE EFFECTS OF YEAST EXTRACTS.

The effect of vitamins on the vitality of protozoa has been a subject of but little investigation. In 1917 Calkins and Eddy (1) reported no effect of treating paramecia with pancreatic vitamin extracted with Fuller's earth. In 1918 Lund (2), working with yeast extracts, found that if *Paramecia* had been starved before being fed with boiled yeast their size and speed of oxidation were increased but not the cell division. In 1919 Chambers (3) reported a slight increase when the animals were fed yeast, especially ground yeast, and in the same year Flather (4) obtained similar results with the unpolished rice. All of these experiments were upon *Paramecium*, which is not a favorable subject for investigation unless pure lines are established and endomixis watched, for a change in the vitality may be due to a reorganization of the protoplasm rather than to a change in the environment. Abderhalden and Kohler in 1919 (5) reported a slight stimulation of *Colpoda cucullus* when treated with yeast extracts but the evidence is not decisive.

Through the courtesy of Professor W. H. Eddy and Dr. Ralph Kerr, of Teachers College, I have had placed at my disposal the following yeast extracts. (1) Alpha bios No. 223 extracted by Professor Eddy in 1924 (6). (2) Beta bios isolated in 1928 by Dr. Kerr (7). (3) Gamma bios a residue substance similar to bios II reported by Lucas and Miller (8) in 1924. These three substances were prepared as indicated in Table I., which has been compiled by Dr. Kerr.

TABLE I.

THE SEPARATION OF YEAST AUTOLYZATE INTO VARIOUS BIOS FACTORS.

I. *Preliminary Fractionation.*

1. Make autolyzed yeast 66 per cent. alcoholic by volume to precipitate proteins. Filter.
2. Filtrate from 1. Add hot saturated baryta so long as an immediate precipitate

- forms. Add alcohol as necessary to maintain 66 per cent. strength. Filter. Save ppt. for 8.
3. Filtrate from 2. Contains alpha bios and some gamma bios. Neutralize immediately with sulfuric. Adjust to pH 4.7. Precipitate with iron sol. and discard pptate.
 4. Filtrate from 3. Adjust to pH 5.3. Precipitate with iron sol. Filter. Save filtrate for 7. Precipitate contains all the alpha bios.

II. Isolation of Alpha Bios.

5. Ppt. from 4. Work up with water and refilter to remove water washings. Dissolve ppt. in 30 per cent. sulfuric. When solution is complete dilute with water and neutralize with baryta. (Fe, SO_4 , ions removed as $\text{Fe}(\text{OH})_3$ and BaSO_4). Filter by suction and discard ppt. With baryta and sulfuric remove quantitatively all Ba, Fe and SO_4 ions.
6. Filtrate from 5. Evaporate to dryness and recrystallize from hot 95 per cent. ethyl alcohol. Purified product has melting point 223°C .

III. Concentration of Gamma Bios.

7. Start with filtrate from 4. Evaporate to small volume. Add sulfuric to make 5 per cent. by weight. Filter if necessary and discard ppt. Now add phosphotungstic dissolved in 5 per cent. sulfuric so long as any ppt. forms. Filter and discard filtrates. Ppt. contains gamma bios.
15. Start with phosphotungstates from 7 and 12. Decompose with baryta in the usual way. Filter. Make filtrate decidedly alkaline to litmus. Add alcohol to 80 per cent. by volume. Filter and discard ppt. if any. Free filtrate of Ba and SO_4 quantitatively. Filtrate now contains a product not yet purified but which suggests Miller and Lucas' bios II. We designate it here as gamma bios.

IV. Isolation of Beta Bios.

8. Start with ppt. from 2. Wash with alcohol. Then stir washed ppt. repeatedly with water and filtrate by suction so long as the water is colored. Neutralize the filtrates immediately with sulfuric. Refilter and discard pptates and residue.
9. Filtrate from 8. Treat with hot saturated Ag_2SO_4 . Filter and discard ppt.
10. Filtrate from 9. Treat with hot saturated acid mercuric sulfate. Filter. Discard ppt.
11. Filtrate from 10. Free from Ag and Hg ions with H_2S . Remove excess SO_4 with baryta. Save clear filtrates. Evaporate at 40°C . to small volume.
12. Filtrate from 11. Make 5 per cent. sulfuric by weight and extract five times with ether of equal volume discarding ether extract. Add phosphotungstic acid in 5 per cent. sulfuric until no further ppt. forms. Filter. Add the ppt. to 7. (See III, 7 above.) Make filtrate slightly alkaline with baryta and refilter. Discard this ppt.
13. Filtrate from 12. Neutralize with sulfuric and evaporate to small volume. Make 80 per cent. alcoholic by volume and again ppt. with baryta. Filter and discard filtrate.

14. Precipitate from 13. Free of Ba with sulfuric. Evaporate to a thick sirup at 40° C. Dehydrate by stirring and grinding with dry acetone to a fine white powder. Filter nearly to dryness on suction filter but leave enough acetone to make a moist mass. Transfer acetone-wet product to vacuum desiccator and here free of acetone by suction. Product is *Beta bios*.

I wish to express my appreciation to Professor Eddy and Dr. Kerr for their interest and helpful suggestions.

The work with the bioses was begun in 1926-27 at the same time when experiments with di-sodium phosphate were being conducted on *Uroleptus mobilis* in order to determine any variations in response according to the age of the protoplasm. Since then *Dallasia* from pure lines of Professor Calkins, *Stylonychia*, and *Pleurotricha* have been used in addition to *Uroleptus mobilis*. Whenever possible the material was taken from pure lines started from an exconjugant or cyst so that the age of the protoplasm was known. In the case of *Stylonychia* conjugation did not occur and the material was obtained from a single individual isolated from the wild culture. The methods used in all experiments were the same as those of earlier papers and as usual the rate of division is considered an indication of the vitality of the protoplasm.

In earlier papers (9, 10), results of experiments have been reported which indicate that the protoplasm of *Uroleptus mobilis* varies in its response to treatment. Beef extracts and di-potassium phosphate cause an increase in the division rate only when the protoplasm is mature while di-sodium phosphate causes an increase in the division rate of cells of all ages but the greatest increase occurs in the mature cells. Experiments with di-sodium phosphate have been continued and will serve as an additional control in the majority of the experiments with the bioses.

I. *Experiments with Alpha Bios.*

Three series of *Uroleptus mobilis* were used in these experiments. Various concentrations of alpha bios were tried and finally a concentration of .05 mg. per cc. was fixed as the best. The bios solution was added to the normal hay flour medium daily and controls were carried on in the normal medium and also parallel experiments were conducted at the same time with a medium containing di-sodium phosphate in the Packard (11) concentration of $M/7000$. The results are shown in Table II.

TABLE II.

EFFECTS OF DI-SODIUM PHOSPHATE AND ALPHA BIOS ON THE DIVISION
RATE OF *Uroleptus mobilis*.

Series No.	Age in Gen.	Amount of Variation from Control in Division Rate per Line in 10-day Periods.	
		Di-sodium Phosphate Series.	Alpha Bios Series.
139	19	+ 3.8	—
	64	+ 3.4	—
	125	+ .6	- 3.0
	175	+ .6	- 2.4
	225	+ 4.4	+ 3.0
	240	+ 1.6	- 2.6
	275	+ 3.4	+ 3.0
140	19	+ .4	—
	60	+ 2.4	+ 1.8
	117	- 3.0	- 3.2
	157	- 1.0	- 4.8
	218	+ 3.4	+ 2.0
	227	+ 4.2	+ .6
141	6	+ 1.2	- 1.8
	36	- 1.0	- 2.2
	66	+ 1.8	- 2.2
	125	+ 1.6	- 2.4
	180	+ 4.2	+ 1.9
	192	+ 4.4	+ 2.4
	234	+ 3.2	+ 4.0
	243	- .6	- 4.0

As in former experiments the sodium phosphate caused a stimulation of the vitality throughout the life history of Series 139 and practically throughout the life of Series 141. Series 140 was the least vigorous and died out in the 227th generation after showing instability throughout its life. The greatest stimulation however in all three series appeared during maturity.

Alpha bios failed to act as a stimulant save in the 225th and 275th generation of Series 139, three times at slightly irregular intervals in Series 140 and only slightly after the 180th generation in Series 141 save in the 234th generation when there was a slightly higher division rate than that of the control or sodium series. Thus alpha bios with few exceptions has a depressing effect on the vitality of these three series of *Uroleptus*.

These results may be due to at least two factors: (1) too acid a

condition of the medium, (2) a general lowering of the vitality of *Uroleptus mobilis*. Undoubtedly the protoplasm was weakening as it did not respond as vigorously to sodium stimulation as it did in the experiments of 1926. On the other hand the H ion concentration of the alpha bios medium was slightly lower than that of the normal medium (7.2). Since trial experiments with beta bios known to be more acid, resulted in a decided lowering of the vitality and since it seemed uncertain whether any bios would cause a definite stimulation of an animal cell, experiments were conducted in which a yeast extract containing all the bioses was used and in one series di-sodium phosphate was added to the yeast extract medium to increase the alkalinity.

II. Experiments with Harris Yeast Extract.

Four experiments with *Uroleptus* at varying ages and two with *Dallasia* were carried on in which the animals were kept (1) in a normal medium to which was added daily yeast extract of a concentration of .01 mg. per cc., (2) in a normal medium to which was added di-sodium phosphate, (3) in the same medium as in (2) with the addition of the yeast extract and (4) in normal hay flour medium as the control series. These results are shown in Table III.

TABLE III.

EFFECTS OF YEAST EXTRACT ON THE DIVISION RATE OF
Uroleptus mobilis AND *Dallasia*.

Series No.	Age in Gen.	Amount of Variation from Control in Division Rate per Line in 10-day Periods.		
		Sodium Phosphate Series.	Yeast Extract Series.	Sodium Phosphate Yeast Extract Series.
<i>Uroleptus</i>				
146....	15	died	— .2	died
143....	30	— 2.0	— 3.6	+ .4
142....	84	— 3.4	— 2.8	— 2.6
141....	250	0.0	— 5.2	— 2.8
<i>Dallasia</i>				
1.....	75	— 3.4	+ 5.0	+ 9.0
2.....	75	+ 3.2	+ 7.2	+ 10.4

The experiments with *Uroleptus* show practically no stimulating effect of the yeast extracts. The protoplasm was too weak to respond and the entire race died out shortly afterwards. The two experiments with two different series of *Dallasia* both in the 75th generation, showed a definite increase in division rate in all the experimental series. It was especially marked in the yeast sodium hay-flour medium when the rate was 10.4 divisions higher than that of the normal control series and 12.4 higher than the sodium hay-flour series for the same period. Since the yeast extracts caused a marked stimulation of the vitality of *Dallasia* both with and without the addition of sodium to the normal medium the fractional extracts of the yeast were then used.

III. Experiments with a Neutral Salt of Beta Bios and with Gamma Bios.

Dallasia, *Pleurotricha* sp. and *Stylonychia* sp. were treated with the two bioses using the same methods as above. These three animals differ in their normal rate of cell division. *Dallasia* when young undergoes from 3-5 divisions daily, *Pleurotricha* like *Uroleptus* not more than 1-2 divisions and *Stylonychia* divided every other day. *Stylonychia* may have been more mature as in this series no conjugations occurred and the age is unknown. The results of the experiments are shown in Table IV.

TABLE IV.

THE EFFECTS OF BETA AND GAMMA BIOS ON THE DIVISION RATE.

Series No.	Age in Gen.	Amount of Variation from the Control in Division Rate per Line in 10-day Periods.	
		Beta Bios Series.	Gamma Bios Series.
<i>Dallasia</i> 2.....	115	- .8	+ 4.8
	130	+ .2	+ 4.8
<i>Pleurotricha</i>	90	+ 5.0 (1st 10 days) died (2d " ")	+ 10.2 (1st 10 days) + 1.6 (2d " ")
	110	—	+ 4.8
<i>Stylonychia</i>		—	+ 3.8 (1st 10 days)
		—	+ 4.8 (2d " ")

Beta bios apparently had no effect on *Dallasia* when older gamma bios, however acted as a definite stimulant increasing the division rate, 4.8 divisions per line in 10 days. *Pleurotricha* was stimulated in the 90th and 110th generation and showed a marked response to gamma bios. *Stylonychia* had its division rate almost doubled in the gamma bios solution and this effect continued for twenty days. When *Pleurotricha* was stimulated for twenty days the effect died out during the second ten-day period. This may be correlated with the variation in normal vitality of the two species.

IV. Experiments with Alpha, Beta and Gamma Bios.

Finally experiments were conducted to compare the effects of the three bioses on *Pleurotricha* and *Stylonychia*, when added to the normal medium and in a few experiments to the sodium medium. The results are shown in Table V.

TABLE V.

EFFECTS OF ALPHA, BETA, AND GAMMA BIOS ON THE DIVISION RATE OF *Stylonychia* AND *Pleurotricha*.

Amount of Variation from the Control in the Division Rate per Line in 10-day Periods.						
Alpha Bios Series.	Beta Bios Series.	Gamma Bios Series.	Na ₂ HPO ₄ Series.	Na ₂ HPO ₄ Alpha Bios.	Na ₂ HPO ₄ Beta Bios.	Na ₂ HPO ₄ Gamma Bios.
<i>Stylonychia</i> + 1.4	+ 4.2	0.0	+ 4.4	—	—	+ 3.2
<i>Pleurotricha</i> 115 gen +10.2	+ 11.4	+ 10.2	+ 8.2	—	—	+ 16.0
130 gen. died..	+ 4.8	+ 8.0	— .8	died	9.0	+ 12.8

In these experiments, *Stylonychia* quickened its division rate in all media save that of the Gamma Bios, where the division rate equalled that of the control. *Pleurotricha* in the 115th generation showed a definite stimulation in all media, especially in that with the HNa₂PO₄ and gamma bios. In the second experiment the division rate of the control dropped to 5.4 divisions per line in 10 days while the experimental series kept a much higher level, the climax being reached with 18.2 divisions per line for the same 10-day period in the series kept in normal

medium to which HNa_2PO_4 and Gamma Bios solution had been added.

SUMMARY AND CONCLUSIONS.

These preliminary experiments indicate that while the protoplasm of *Uroleptus mobilis* was usually depressed when treated with yeast extracts due probably to its weakened condition, that of *Dallasia*, *Pleurotricha* and *Stylonychia* were definitely stimulated by the addition of fractional extracts of yeast to the normal medium. Alpha bios in general causes the least effect and gamma bios the greatest increase in division rate. While there is usually an increase in the protoplasmic activity when sodium phosphate is added to the medium already containing the bios solution, this may not be due to an increased alkalinity as the variation in Hydrogen ion concentration were not more than .1-.2 of a point. The explanation may lie in an increase in the permeability of the cell allowing a far more reaching effect of the bios solution. The age and general characteristics of the protoplasm must also be taken into consideration and further experiments are planned with pure lines of varying ages.

The fact of a sudden marked increase in the division rate of a protozoan cell when treated with these yeast extracts brings to mind the theory of Burrows in which the rapid growth of cells and formation of tumors is assumed to be due to a lack of balance between vitamins in the cells. The relation of these extracts to vitamins has yet to be proved. They are however stimulating substances to yeast cells and to certain animal cells, their effects varying according to the age and general conditions of the protoplasm.

BARNARD COLLEGE,
June, 1928.

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BIOLOGICAL BULLETIN

INSECT METABOLISM.

THE ANAËROBIC METABOLISM OF AN INSECT (ORTHOPTERA).

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That insects can live anaërobically for varying periods of time has been repeatedly pointed out (Winterstein, 1921; Lee, 1924, 1925; Willis, 1925; Davis and Slater, 1926, etc.). When deprived of oxygen they enter into a state closely resembling anesthesia. Various methods for the withdrawal of oxygen have been experimentally employed, such as replacing the air by the gases hydrogen, carbon dioxide, nitrogen, etc.; by evacuating the vessel in which the insects are contained and by immersing the insects in water. The results produced by all of these methods closely resemble each other and if the deprivation of oxygen has not been too long the insects recover and appear quite normal. A state of anaërobiosis thus produced in insects offers rather unique conditions for studying the gaseous exchange of an organism during oxygen lack.

The present work deals with the rates of oxygen consumption and the blood pH changes in grasshoppers under normal as well as anaërobic conditions.

MATERIAL AND METHODS.

The grasshoppers, including individuals of the following species, *Melanoplus differentialis*, *Melanoplus femur-rubrum*, and *Chortophaga viridifasciata*, were hatched and raised under laboratory conditions and fed lettuce. Organisms of known and varied ages were used in the experiments. Oxygen determinations were made by means of the modified Krogh Manometer

(Bodine and Orr, 1925), immersed in a Freas constant temperature water bath maintained at $25^{\circ} \pm 0.1^{\circ} \text{C}$. Animals were subjected to anaërobic conditions largely by immersion in water at 25°C . for varying periods of time. Immersion in water was found to give results identical with those produced by the gases hydrogen, carbon dioxide or nitrogen.

Animals were first put in the manometers and their normal rates of oxygen consumption determined. After removal from immersion in water they were again quickly put in the same manometer and their rates of oxygen consumption during recovery noted. By such a procedure a continuous record of the oxygen consumption of the organism was obtained except for the actual period of immersion in water.

Blood pH determinations were made by micro-colorimetric methods (Bodine, 1925). By means of fine capillary pipettes blood was easily obtained from minute punctures made by fine needles in the lateral abdominal wall of the animal.

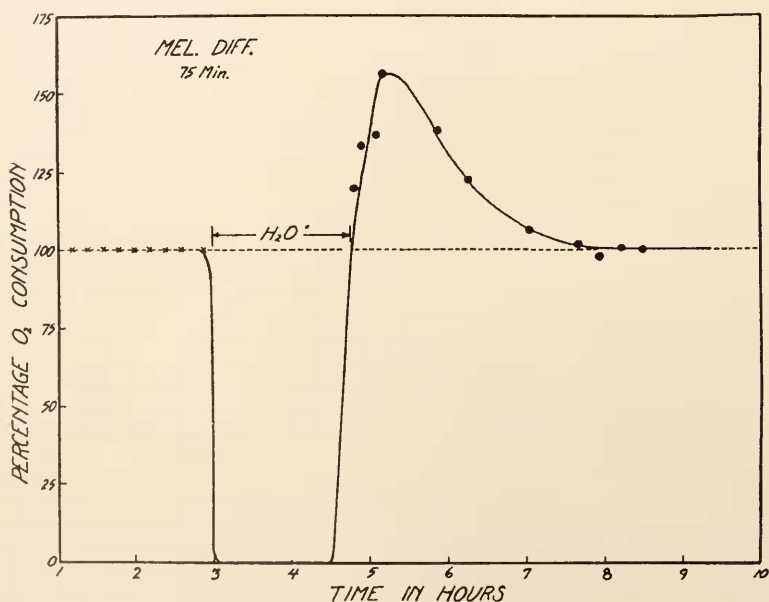


FIG. 1. Curve showing the effect of immersion in water for 75 minutes on the rate of oxygen consumption of a male, nymph, *Melanoplus differentialis*. Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

RESULTS.

Oxygen Consumption.

The normal rate of oxygen consumption for each organism was determined over a period of an hour or more until a constant rate was obtained. The animal was then removed from the manometer, placed in a glass tube, the open ends of which were covered with wire gauze, and immersed in water at 25° C. to a

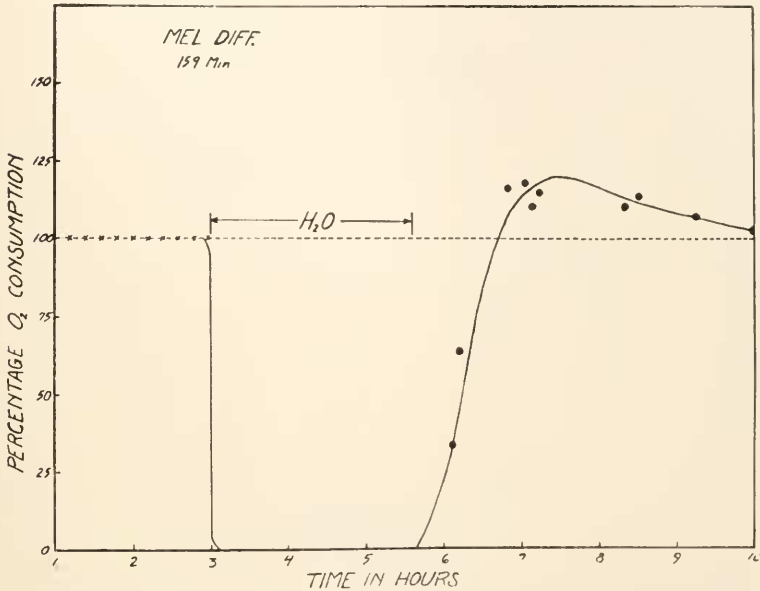


FIG. 2. Curve showing the effect of immersion in water for 159 minutes on the rate of oxygen consumption of a male, nymph, *Melanoplus differentialis*. Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

depth of 180 mm. All air bubbles were removed from the surface of the animal and the ends of the tube by gentle shaking. The animal becomes motionless within a very short time after immersion and remains so throughout the immersion period. After immersion, the organism is quickly removed from the glass tube, dried on filter paper and returned to the same manometer originally used to determine its normal rate of oxygen consumption. The recovering animal is left in the manometer and its rate of oxygen consumption followed until complete recovery

occurs. Since the general response of all organisms is essentially the same, only typical experiments will be presented.

Figures 1, 2, 3 and 4, in which the rates of oxygen consumption are expressed in terms of the normal rates (100 per cent.), show graphically the changes in the rates of oxygen consumption produced by exposures to lack of oxygen. An examination of these

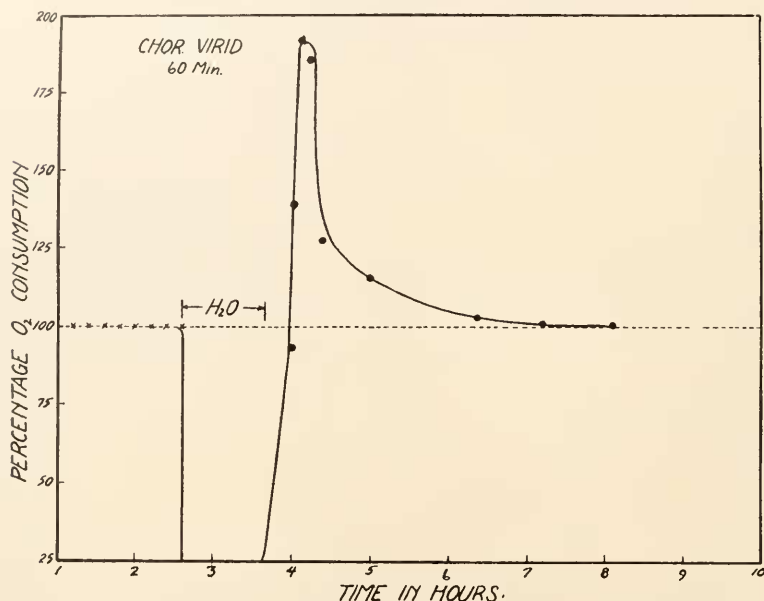


FIG. 3. Curve showing the effect of immersion in water for 60 minutes on the rate of oxygen consumption of a male, nymph, *Chortophaga viridifaciata*. Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

figures further shows that when the animal is readmitted to oxygen after immersion its rate of oxygen consumption increases considerably over the normal rate or that characteristic for the animal before deprived of oxygen. This excess oxygen taken up by the organism can be shown, in carefully controlled experiments, to be approximately equal to the amount the organism would have taken up normally during the period it was deprived of oxygen. In other words, it seems that the grasshopper when deprived of oxygen or existing anaërobically, goes into debt for oxygen in a manner quite similar to that pointed out for the

cockroach (Davis and Slater, 1926) and for heavy physical work in man or for isolated muscle (Hill, 1922).

The length of exposure to lack of oxygen that can be withstood by different species of grasshoppers varies as pointed out below. Some species have been found to successfully withstand as high as 7 hours immersion in water. The rates of oxygen consumption during recovery, as indicated in Figs. 1, 2, 3 and 4,

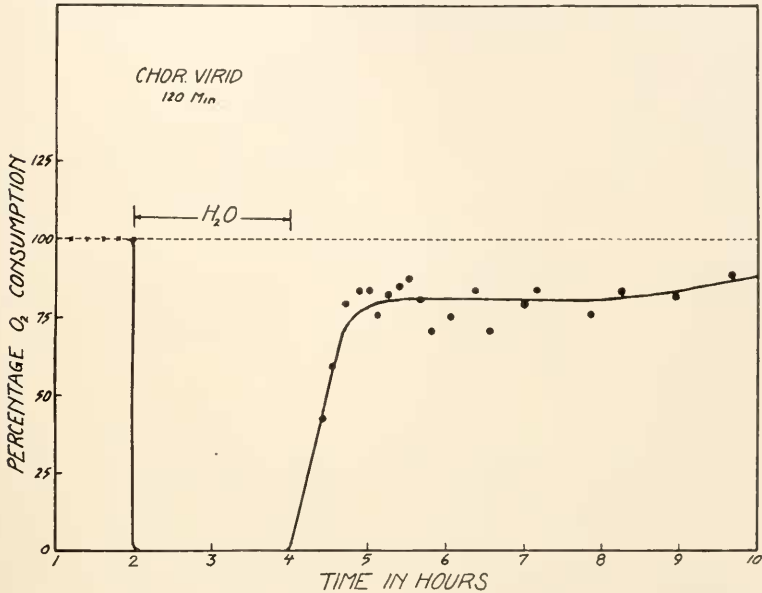


FIG. 4. Curve showing the effect of immersion in water for 120 minutes on the rate of oxygen consumption of a male, nymph, *Chortophaga viridifasciata*. Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

seem to be greatly influenced by the length of the immersion period. Animals immersed for 60 to 120 minutes usually recover in a typical manner as shown in these figures. When the immersion period is lengthened, however, there is a strong tendency for the rates of oxygen consumption to return to normal in an extremely slow fashion as shown in Figs. 2 and 4. This slowness in recovery to a normal rate of oxygen consumption is probably correlated with the physiological condition of the organism as well as with the fact that the exposure might be just a sub-lethal one for the animal.

There also appears to be a marked difference in the rates of recovery in the different species. *Melanoplus differentialis* seems better able to reversibly withstand long immersion than *Chortophaga viridifasciata*, as indicated in Figs. 2 and 4. Age is also an important factor, since younger individuals withstand and recover from long immersions better than older ones.

The relations between length of immersion in water and recovery time for the different species of grasshoppers examined are

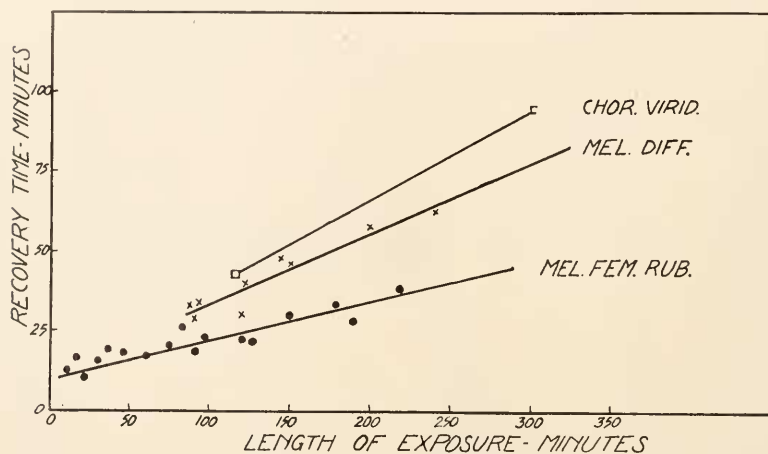


FIG. 5. Curves showing relation between average mean recovery time and length of immersion in water in young adult grasshoppers of three species, *Melanoplus femur rubrum*, *Melanoplus differentialis* and *Chortophaga viridifasciata*. Each curve based on several hundred observations. Recovery time indicates return of "turn-over or righting" reflex.

graphically shown in Fig. 5. From a study of this figure it is evident that a linear relationship exists between length of immersion and recovery time. That the causes of the anaesthetic condition produced by lack of oxygen are doubtless due to the carbon dioxide and lactic acid produced within the organism seems reasonable when a comparison is made between the results obtained by immersion in water and those obtained by subjecting the organism to carbon dioxide (Willis, 1925), as indicated in Fig. 6. These curves are qualitatively similar. In view of similar evidence gained from studies on anaërobiosis of mammalian muscle (Hill, 1922), such an hypothesis seems reasonable in explaining these phenomena in grasshoppers. As a matter of

fact, Lee (1924) has shown that injection of carbonic acid or lactic acid into a grasshopper produces results quite similar to the present ones on immersion of the animals.

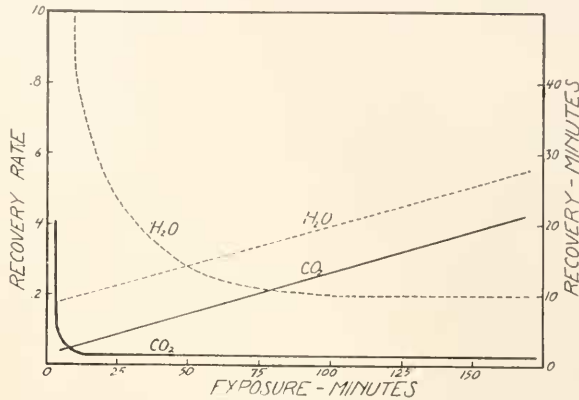


FIG. 6. Curves showing comparisons between average mean recovery times and rates and length of immersion in water and exposure to carbon dioxide in *Melanoplus differentialis*. Carbon dioxide data from Willis (1925).

BLOOD pH.

As previously pointed out the normal pH of the blood of the grasshopper, *M. differentialis*, seems to be 6.8. (Bodine, 1926.) A careful study has been made of the blood of individuals throughout the present immersion and recovery experiments. Fig. 7, in which are plotted together results of experiments carried out for different periods of time, shows graphically the pH changes occurring in the blood during the anaërobic and recovery periods. A progressive lowering in pH values with length of immersion occurs and seems to reach a minimum at about 5.8. Below this minimum the animal seems irreversibly affected. Upon recovery, a slow blowing off of acids (H_2CO_3), probably occurs and the blood then gradually returns to its normal pH value. An interesting fact, indicated in Fig. 7, is that no marked changes in blood pH occur in immersed animals when removed from the water until after respiratory movements have become established. Upon careful examination of the animal it is found that initial respiratory movements upon recovery are extremely slow and of great depth. As recovery progresses the movements become more regular and normal. These changes in blood pH strongly

suggest that during anaërobiosis large quantities of acids, carbonic and lactic, are produced and that recovery consists largely in their elimination by the organism.

As pointed out by Davis and Slater (1926), who have found similar results for the anaërobic metabolism of the cockroach, insects seem to be extremely favorable material with which to further elucidate the changes occurring during the anaërobic existence of organisms and the question of energy formation under such conditions.

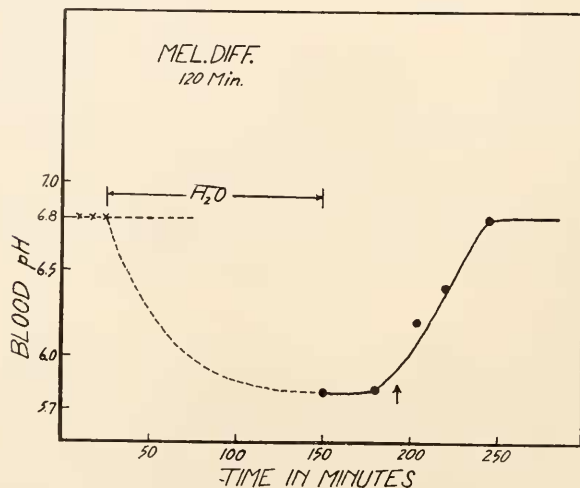


FIG. 7. Curve showing the effect of immersion in water for 120 minutes on the pH of the blood of a male, nymph, *Melanoplus differentialis*. Space within arrows indicates period of immersion of the animal; points on curve, actual pH determinations of blood before and after immersion. Broken portion of curve is composite, being made up from pH determinations of the blood of individuals immersed for periods of time ranging from 50 to 120 minutes.

SUMMARY AND CONCLUSION.

1. Rates of oxygen consumption in grasshoppers before and after immersion in water (lack of oxygen) have been determined.
2. During oxygen lack, grasshoppers build up an oxygen debt. When readmitted to oxygen an increased rate of oxygen consumption occurs and an excess of oxygen, approximately equal in amount to that which the organism would have taken up normally during the period it was deprived of oxygen, is consumed.

3. During anaërobiosis blood pH falls. Upon recovery pH values slowly return to normal.
4. It is suggested that the chemical change responsible for the anesthetic condition accompanying anaërobiosis is the production of an excess of acid, carbonic and lactic, and that recovery consists in their elimination.

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THE PULSATORY RHYTHM OF THE CONTRACTILE VESICLE IN *PARAMECIUM*.

FRANCIS E. LLOYD AND J. BEATTIE.

I.

It became apparent to the senior author a year ago that there are discrepancies between the more recent, and therefore presumably the most correct, accounts of the behavior of the contractile vesicle in *Paramecium* and the objective facts as apprehended by him. As to these facts both the present authors have found themselves in agreement, and it was therefore determined to make an investigation of said behavior by such refined means, by way of control of direct and unaided vision, as are available. Two methods were used, that of recording visual observations of critical points in the contractile cycle on a rotating drum and, still better, that of making motion pictures at normal speed, viz: 16 per second, without any lapse. It is not easy to get a *Paramecium* to remain within the field of vision long enough to take a motion picture so as to get a continuous view of the contractile vesicle for two or three cycles of movement. We have however succeeded by making use of very slight compression between cover slip and slide—a method to which there is some, but we believe not wholly justified objection—and by surrounding the animals with a suspension of Chinese ink, a time honored method for demonstrating the expulsion of fluid from the contractile vesicle, as used by Carter (1861) and by Jennings (1904) and by many others before and since. It happens that a rather thick suspension of this insoluble pigment impedes the movements of the animal, so that the chances for observing a relatively quiet one, with the contractile vesicles in full view, is much increased without, we think, in the least affecting the behavior of the vesicles themselves. We suspect that in Chinese ink there is an admixture of some aromatic substance which may act as a mild depressant, narcotic or otherwise, but of this we have no sure knowledge. However, the animals can live for several hours

under a cover-glass in a thick suspension of Chinese ink and appear quite undamaged. We have no doubt at all events that the behavior of the vesicles is normal. The motion pictures produced by the senior author have been shown ¹ in illustration of this paper; in the accompanying plate we present a few excerpts from one of the films to serve present purposes.

The point of departure of this study may be better appreciated by considering in the first place the latest pronouncement on the subject, that of Nasonow (1924). In the text which follows we shall speak simply of the vesicle ² (= contractile vacuole) and the canals (= rays, canaliculi, radial canals). The general topography of the apparatus is so well known that we may take familiarity with it for granted. Nasonow says:

"After the emptying and the complete disappearance of the vesicle there become visible in its immediate vicinity the 5-7 canals. The ends directed toward the center of the vesicle are strongly swollen and no continuity between them is to be seen. The swollen ends approach each other, flow into each other and form a new vesicle, into which the fluid of the canals now flows. Thereupon the canals quite disappear and only later do new canals begin to be formed in their place the ends of which after the emptying and disappearance of the vesicle suffers enlargement and in this manner complete the cycle" (Nasonow, 1924,³ p. 454). Nasonow then goes on to recall the views of others, including that of Stempel (1914) in regard to the existence of a membrane, with which Nasonow was particularly concerned, apparently acquiescing completely with this author in respect of the progress of the cycle of behavior. We therefore quote Stempel also as follows:

¹ Winnepeg meeting of the Royal Society of Canada, May, 1928.

² It seems to us that Claparede and Lachmann chose the better terminology, and we follow them, with Pritchard.

³ Among the figures illustrating the paper by Nasonow occur two which we may remark in passing to be capable of precisely the opposite interpretation to that given by him. His Fig. 40 is labelled "Diastole of the excretion apparatus" while Fig. 42 is labelled "Systole of the excretion apparatus." If by excretion apparatus he means the vesicle then figure 40 represents early systole and figure 42 early diastole. If however he means the canals then his labelling is correct. It is not easy to understand his exact meaning. It is certain that diastole and systole of the vesicle are not synchronous with those of the canals.



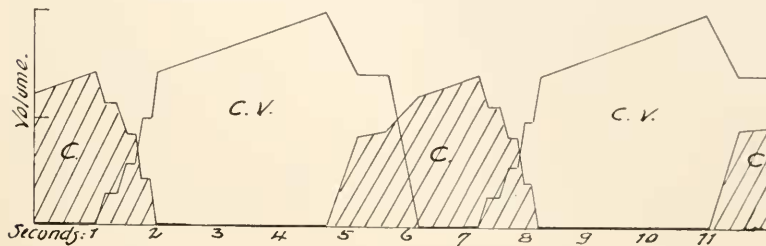
"The end-products of metabolism collect in dissolved condition in certain places in the protoplasm, namely, in a canal- or space system (probably a branched one) the exits of which run towards the two pulsating vesicles as afferent canals. As soon as the vesicle is emptied ('Sobald die Vacuole sich entleert') the ends of these afferent canals swell up to form 'Bildungsvacuole' since the fluid flows hereinto as to the place of minimal pressure, and is here dammed up. As a result of this pressure delicate protoplasmic valves open and permit the volumes of fluid which have collected in the canal-ends to flow together in the vesicular space, on which, after this is filled, the valves promptly close. Since the fluid now collected in the vesicle has a high osmotic pressure, there results a lasting addition of water to the fluid already held in the vesicle by diffusion through the semipermeable membrane formed *ad hoc*. As soon as the pressure of the fluid in the vesicle has reached a certain height, that is, has become higher than that of the external water pressure, a second valve at the apex of the papilla-like dome of the pellicula opens and there results from the pressure of the protoplasm and of the surface tension of the vesicular drop a complete emptying of the vesicle, whereupon the process is repeated in the same way" (Stempell, 1924, p. 460). Here it may be remarked that Fortner (1926*a* and *b*) and v. Gelei (1926) without inquiring into the validity of the above view, proceeded on the assumption of its truth.

With regard to Stempell's ideas, as above expressed, there can be no doubt since he has furnished us with a diagram. From both description and diagram we learn that Stempell does not entertain at all the idea that there is any *flow of fluid from the vesicle into the canals*. Nassonow's idea is identical, we believe we are right in saying, but his diagram, taken from Pütter (1903), might be interpreted otherwise, as witness Figs. 5, 6 and 7, which show the canals enlarging before the contraction of the vesicle. Whether this enlargement results from backflow from the vesicle, or from the collection ("ponding back" as Carter expressed it) of fluid derived from the surrounding protoplasm, is the question with which we are concerned. We are now in a position in the second place to examine the view of earlier observers of the same phenomenon.

Felix Dujardin (1841) made a drawing, reproduced in his Plate

8, Fig. 6a and 6b in which the radiating vesicles ("taken by Ehrenberg for seminal vesicles") are seen in the condition just before the systole of the vesicle, the canals being expanded. Dujardin does not, of course, have anything to say about the matter, but his drawing could not be correct if the canals do not fill before the systole of the vesicle. The question above indicated therefore recurs, whence the fluid which fills the canals.

As to this there was no doubt in the minds of Claparède and Lachmann (1858) (Lachmann 1857 for 1856). It was these who held the view that the apparatus is the homologue of the circulatory apparatus in the more differentiated animals and



it was consistent with their view that there was no opening affording an exit for the fluid to the outside. Their unfortunate error in this seems to have led to a general condemnation of their whole conception and thereby their critics, in overlooking what they did see, fell into an error as grievous, namely, in failing to see that the canals are in the first instance filled at the expense of the vesicle, as we hold. Lachmann's description (1857) will serve our turn at the moment.

He says (1857, p. 224), maintaining that the thin area of the body wall over the vesicle is only a thin place fit for diffusion and with no opening, that when the vesicle is fully expanded the canals are fine lines. By the sudden contraction of the vesicle, however, the canals instantly swell into pyriform spaces close to the contractile vesicle, which has disappeared. During the slow reappearance of the vesicle, the canals gradually decrease and they have again been reduced to fine lines by the time the vesicle has become fully inflated.

It must be clear that Lachmann believed that the swelling of the canals is synchronous with the early period of systole of the

vesicle. Carter (1861) does not, we think, correctly take his meaning when he says: "Claparède and Lachmann have said that the fluid of the vesicle is returned into the vessels on the systole or contraction of the vesicula because the sinuses and vessels become filled *immediately afterward*" (italics ours) as this is not what Lachmann said.¹

It is in this connection that Carter suggests that the swelling of the canals into the characteristic pyriform is due to the "ponding back" of the fluid which flows through the canals into the vesicle for the short time that the latter empties itself, like the ventricles of the heart but in the other direction (1861, p. 282). We may here remark, what we shall endeavor to show to be true, that the rate of swelling of the canals does not consist with the idea that the fluid reaches the lacunæ by diffusion through the walls, the rhythm of diastole and systole in these being of the same character as in the vesicle; and, if the rhythm of the vesicle can be understood only when it is admitted that the fluid of the canals gushes into it, the same must be admitted for the canals, but in the opposite sense.

Somewhat earlier, and in contrast to Lachmann and Claparède Lieberkühn (1856), while agreeing with them as to time relations of vesicular systole and canalar diastole, saying that "a little before we observe the commencement of the systole, the vessels begin to expand slowly, etc.," simply denied that there is any backflow. The interest here is obviously the correct observation in regard to time relations in question. Spallanzani also believed that the canals become empty as the vesicle fills, and do not reappear until some time after it has contracted and that therefore "The fluid with which the vesicula is distended comes through the sinuses, but is not returned by them to the body" (through Pritchard, 1861).

J. Müller (1856) appears, according to Claparède and Lachmann (1858, p. 51), to have taken the same view of the time relations. We transcribe their summary of his views, since we have been unable yet to see Müller's original paper. This author distinguishes in the behavior of "central circulatory apparatus" of *Paramecium* two partial systoles which alternate with each

¹ We have not been able to see Claparède's paper, but it appears that these two observers, Claparède and Lachmann worked in harmony, sharing each other's views.

other—the systole of the vesicle, then the systole of the fusiform or pyriform swellings. The latter coincides with the diastole of the vesicle. Lieberkühn had already observed that “un instant avant le systole des vesicules les rayons se renfluent considerablement.” Müller explains the phenomenon by showing that the vesicle contracts, diminishing insensibly in volume in the instant which precedes systole and forces at once a part of its contents into the “rays of the star.” Then the systole of the vesicle takes place, which produces a further swelling of these rays.

We cannot refrain from mentioning, in passing, the work of Wrzesniowski (1869), who studied *Enchelyodon*, *Trachelophyllum* and *Loxophyllum*, (but was however chiefly concerned with the question of the absence or presence of a contractile membrane), because there is some evidence in his results which point to the presence of a contractile vesicular apparatus similar to that of *Paramecium*, though the author himself, if he adhered to the original account, would deny this. One point may be mentioned, however, namely, that a series of small vesicles is formed on the surface of, and from the contractile vesicle during early systole, and these, upon growth, run together later to form a new contractile vesicle (not the old one reëxtended). This view of Wrzesniowski's seems to be strongly linked with his conviction that the vesicle is formed *de novo* and totally lacks a membrane in any but the sense of molecular physics as Khainski (1911) would express it.¹

We pass to the year 1883 when Maupas attacked the subject. According to him the systolic movement of the vesicle is sudden and rapid. A little before it happens the canals commence to fill in the form of elongated pears at a little distance from the point where they open into the vesicle. Maupas' account indicates a high degree of meticulous care in observation. He goes on to remark for example that the systole of the vesicle takes place more often before the pyriform swellings (of the canals) have attained their full size. In spite of the fact that he correctly apprehended the time relations involved he pronounces for the view that the canals are simple afferent conduits and sententiously

¹ Samuelson observed in 1857 that the single globular vesicle in *Glaucoma scintillans* when it contracts forces the fluid into others which appear temporarily around it.

remarks "I have never seen the liquid of the vacuole reënter them." This would indeed be difficult and his failure cannot be charged to his discredit, for at all events he very correctly describes the at first irregular contours of the vesicle during the early stages of its diastole when, under systole of the canals, these empty themselves into the vesicle. Maupas was on the side of the non-membranists.

It will be seen that these earlier observers, while disagreeing in regard to the afferent-efferent nature of the canals, support a majority view which, as we believe, correctly describes the time relations between the behaviours of the vesicle and the contributory canals. It is therefore a curious fact that later observers, as we have already shown at the outset of the paper, siding with the view of the solely afferent nature of the canals have in some way been led to overlook the true time relations.

Closely connected with the general trend of inquiry above outlined is the parallel inquiry into the nature of the membrane lining the vesicular cavity. It will be easily apprehended that very convincing evidence has been so difficult to obtain that only recently has Miss Howland (1924) favored the view that a proper membrane in the morphological sense is present constituting the branching cavity composed of the central vesicle and its contributory canals. She succeeded in isolating the membrane with little distortion by micro-dissection from an animal (*Paramecium caudatum*) treated with a strong solution of alizarin blue. This author expressed some doubt of her interpretation based on the possibility that the dye had coagulated the surface material of the vesicle and so produced an artefact. In the same year Nasonow presented evidence based on the method of osmication which would convince even the elect were it not for a doubt similar to that expressed by the former author. We venture to think that a weak link exists in the chain of his argument. We are not here concerned with this author's views of the homology of the contractile vesicle with the Golgi apparatus although we subscribe to the general view supported by Nasonow that the pulsating vesicle is a true organelle of morphological value, as Lachmann so long ago held. With regard to earlier observers it will boot us little to bring forward the details of their views, a summary of

which will be found in a paper by Taylor (1923). Fortner, by compressing animals in a hypertonic solution of cane sugar, was able to set free the apparatus surrounded by protoplasm and in a state of approximate diastole. Their behavior he argues unqualifiedly postulates the impermeability of the membranes; but these membranes he believes arise *ad hoc*, that of the vesicle at the completion of each systole affording the new membrane for the papilla pulsatoria. Without further discussion of this matter from the historical point of view we may be permitted to remark that had the true time relations in the cycle of events not been lost sight of, the protagonists of the "non-membranous" view would have suffered pause.

II.

No special technique is required to demonstrate the phasic activity of the contractile vacuole and canaliculi in *Paramecium*. Care must be taken that the cover slip over the preparation does not press untowardly on the animal, otherwise the pore to the exterior may be blocked and the contractile vesicle fail to discharge in the normal manner, and at normal rate.

After the preparation has been made it is well to allow some minutes to elapse before the preparation is examined as it is a hopeless task to attempt to observe the contractile vesicle in one single animal, while the animals are in rapid motion immediately after they have been placed on the microscopic slide. In a short time the animals settle down to feed, and it is then possible to watch a whole group and to pick out one animal for observation. It is possible also to trap the animals in a very fine capillary tube and so limit their movements except round a longitudinal axis. A better method, but open to the objection of an abnormal environment, is to mix finely ground China ink with the mounting medium. This appears to impede the movements and so far as one can see there is no interference with the normal cycle of events within the contractile vacuole system.

After close observation for a few minutes the following series of changes can be seen. The contractile vesicle will be observed as a highly refractile almost spherical droplet lying in the most superficial part of the cytoplasm. When the animal rolls over on its side it will appear that at one point there is a close attachment

of the vacuole to the pellicle. At this time the vacuole when viewed from the side will appear as three quarters of a sphere with a conical apex attached to the pellicle. When the animal rolls so that the vacuole is observed from above with careful focusing a bright minute ring will be seen in the center of a small clear area in the pellicle. This is the pore through which the vesicle expels fluid to the exterior. The vesicle gradually enlarges and in doing so changes its shape from the conico-spherical form to a perfect sphere. Enlargement after the spherical shape has been attained is slow and very small in amount as to linear dimensions. Suddenly at the end of diastole the vesicle appears to get smaller (Plate I, Figs. 3 to 4) and *at the same moment, not afterwards*, radiating canals appear surrounding the vacuole (Plate I, Fig. 3). Seen from above the inner ends of these structures are separated from the vacuole by a distinct area of protoplasm. Seen from the side the bulbous or pear-shaped ends of the canals are observed to lie in the most superficial layer of cytoplasm and to be continued more distally into the deeper parts of the cytoplasm as fine canals.

After this phase, which can only be interpreted as a distinct diastole of the canaliculi caused by systole of the vacuole and not merely as a damming back of liquid attempting to flow into the vesicle, the vacuole suddenly contracts (Plate I, between Figs. 4 and 5) and expels the remaining contents to the exterior. Therefore systole of the contractile vesicle consists of two distinct phases:

- (a) First, an early systolic phase during which the contractile movement of the vacuole is slow and diastole of the canals rapid (Plate I, Fig. 3, 4).
- (b) Second, a later period during which the vacuole expels the remainder of its contents to the exterior (Plate I, Fig. 5).

The behavior as thus set forth has been displayed graphically in the accompanying diagram, in which, to some extent provisionally, we have attempted to express the time relations seen in the rhythm of the contractions and expansions of vesicle and canals, while the volume relations are avowedly inexact, but approximate. Time is plotted on the abscissa, and the volume of the canals and of the vesicle on the ordinates, the total volume of the vesicle being taken as one. The hatched areas are bounded

by the curve of diastole and systole of the canals; the areas bounded by the curves for the vesicle are left blank.

There is no doubt that there is a discharge of vesicular contents to the exterior. Jennings showed this first convincingly as has been stated above and we have been able to confirm his observations and to make a motion picture of the process.

There has been some doubt expressed as to whether or not the pore through which the vacuole discharges can admit fluid from the surrounding medium. We have found no evidence to support this theory. All our observations go to show that after the vesicle has discharged its contents reconstitution of the vacuolar space takes place by the discharge into the collapsed cavity of the fluid contained in the canals (Plate I, Figs. 1-2; 6-8). Discharge of the contents of the canals into the vesicular space takes place within one second after the completion of systole of the vacuole. The canals do not however discharge simultaneously but by careful observation one is able to make out that first one canal may discharge into the collapsed vesicular region which then forms an irregular angular cavity¹ soon followed by another and then by the remainder. When the last canal has discharged the space is seen to be conico-spherical as described above. It is possible to analyse the discharge of the canaliculi into the vacuolar space only by study of the motion picture film.

After the reconstitution of the vesicle enlargement takes place and this phase of diastole of the vacuole occupies the longest period of the cycle of events. One notices that the conico-spherical form persists for quite a time (almost three quarters of diastole) before the spherical form is assumed. Once the vesicle becomes spherical systole of the structure takes place within a second or two.

The cycle of events occupies normally about eight seconds. Records which we have made show that in fresh specimens cycles of seven and one fifth seconds were common. We have observed cycles which required ten seconds for completion. When the cycle lengthens it is the diastolic period which is chiefly prolonged. When the animal is compressed gently it is possible to occlude the pore and so prevent the second phase of systole taking place. The first phase, *i.e.* diastole of the canals takes place but there is

¹ Beautifully recorded by Nassonow, Fig. 42.



no discharge to the exterior. In a short time the canals reappear and so the cycle goes on. The vacuole continues to enlarge and before very many minutes the pellicle ruptures and the protoplasm is extruded carrying with it in some cases the entire contractile vesicle. The vacuole may be seen lying as a spherical body in the surrounding fluid. We have not observed any sign of a canal when the protoplasm is examined after bursting.

When neutral red is used in solutions of one part to four hundred or higher concentrations it is frequent to observe the gradual contraction of the cytoplasm from the pellicle and the formation of a peri-cytoplasmic space filled with fluid. In one specimen of which a photograph is shown (Plate I, See Fig. 9) the cytoplasm in contracting pulled a fine cone of pellicle downwards. At the apex of this was attached the pulled-out contractile vesicle which extended as a conical cavity through the peri-cytoplasmic space to the dimple in the pellicle. The actual interface between the vesicle and the surrounding fluid could be seen. The apex of the dimple was the pore through which the vacuole discharges. It also would appear to show that the vacuole when it discharges to the exterior is not reconstituted *de novo* in the old site but rather that there is something of a permanent nature—a vesicular membrane into which is discharged the contents of the canaliculi when the vesicle is reformed.

Miss Howland, as we have already said, has been able to isolate the vesicular membrane from preparations treated with alizarin blue.

By way of summary of the above we draw attention to the following important facts.

Diastole of the vesicle falls into two phases—an early rapid and a later slow one.

The early rapid phase is due to the systole of the canals during which their fluid content is forced into the vesicle.

The later slow phase of diastole of the vesicle is due to further distention by diffusion of water into the vesicle.

Systole of the vesicle falls similarly into two phases, an early slow phase during which the fluid is forced into the canals (diastole of these) and a later rapid phase during which the remainder of the vesicular fluid is forced through the spore into the surrounding medium.

It appears that early diastole of the vesicle is synchronous with the systole of the canals; and that early systole of the vesicle is synchronous with diastole of the canals. During early diastole of the vesicle, this is partly filled with fluid from the canals, This is the residual fluid plus that which has in the interim entered by diffusion into them. During early systole of the vesicle the canals are partly filled with fluid from it—this we may speak of as the residual volume. That volume which is discharged by the vesicle is the overplus accumulated by diffusion into the vesicle and canals during their diastolic periods.

Viewed thus, the mechanism is one in which a certain quantity of fluid of relatively high osmotic pressure is retained in the canals, derived by them from the central vesicle, and which is at once put into service to withdraw water from the body into the pulsatory apparatus. Thus an important feature of Stempell's view receives support, even though his conception of the methods of working of the apparatus is incomplete. If it depended solely upon diffusion for filling, from the completely collapsed state to the completely replete, it could, in our opinion not work so rapidly and efficiently.

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EXPLANATION OF PLATE.

FIGS. 1-8. Eight episodes from motion picture (photomicrographic) of *Paramoecium caudatum*. The animal was slightly compressed between slip and cover; the periodicity was slightly slower than normal therefor. The position in the film is indicated for each. Exposure 16 per second. Enlargements at constant distance.

FIG. 1. Foot 887 frame 16. Mid-diastole. Canals are emptying into vesicle.

FIG. 2. 883-1. Late diastole. Traces of canals visible.

FIG. 3. 880-16. Early systole of vesicle; canals beginning to fill.

FIG. 4. 876-10. Mid systole of vesicle which is now smaller; canals nearly filled.

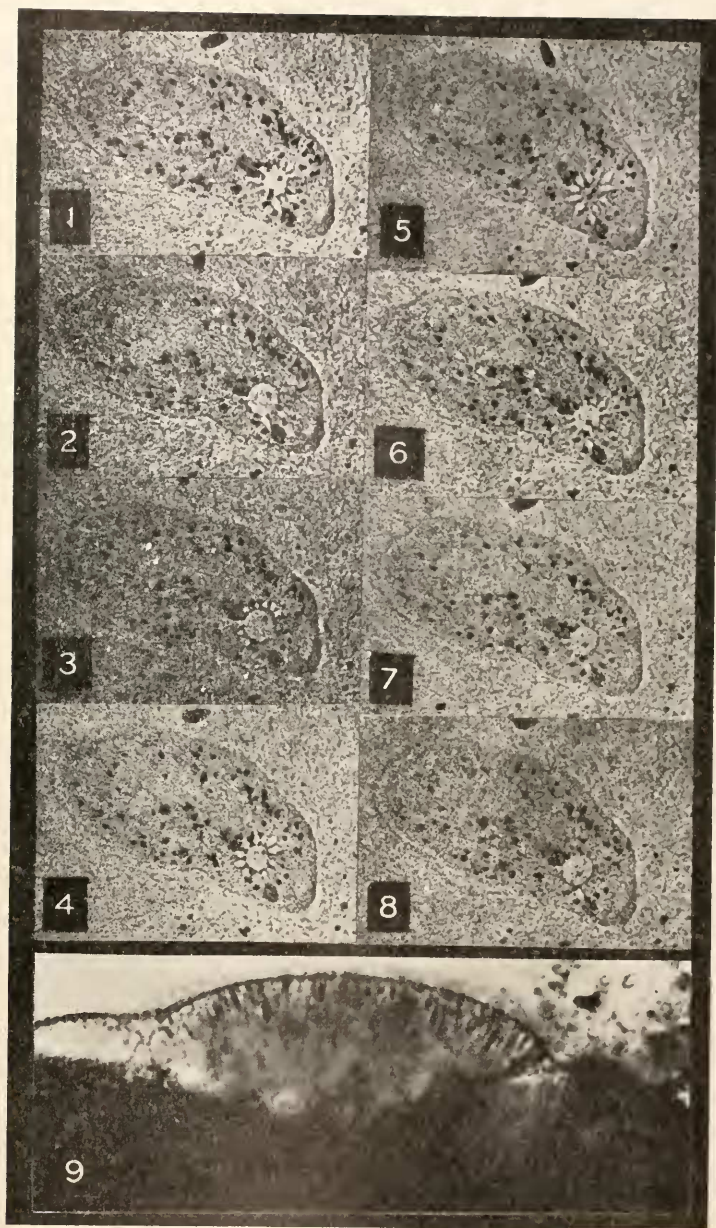
FIG. 5. 873-15. Systole of vesicle complete, canals full.

FIG. 6. 871-10. Mid-diastole (somewhat later than Fig. 1); canals emptying into vesicle.

FIG. 7. 870-3. Later diastole; canals nearly disappeared.

FIG. 8. 868-9. Diastole complete; canals empty. One canal persists longer than the others: note that it occurs in Figs. 1, 2, 5-8.

FIG. 9. An animal treated with neutral red (see text), showing the vesicle pulled away from the pellicle, and dimpling it by pulling on the pore rim. The result follows from the shrinkage of the cytoplasm.



OBSERVATIONS ON *HYDRA* AND *PELMATOHYDRA*
UNDER DETERMINED HYDROGEN ION
CONCENTRATION.

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Much has been written recently concerning reduction, dedifferentiation and resorption in *Hydra*. It is generally conceded that reduction in hydra is accompanied by a loss of tentacles. The literature enumerates the following causes by which hydras lose their tentacles. N. Annandale ('07) observed, in studying *Hydra orientalis*, that during the hot season of the year this species has but four tentacles while during the cold season it has six tentacles. G. Entz ('12) observed that an infection with *Amæba hydroxena* may lead to a degeneration of tentacles. Reynolds and Looper ('28) have come to the conclusion that this parasite is responsible for the degeneration of the tentacles. Certain ciliates recorded by E. Reukauf ('12) and P. Shultze ('13) also caused the loss of tentacles. E. Shultz ('06) observed that hunger set up a process of dedifferentiation within the tentacles. Huxley and DeBeer ('23) observed that adverse environmental conditions accelerate dedifferentiation and resorption of the tentacles of *Obelia* and *Campanularia*. They also found that this process of dedifferentiation and resorption might involve not only the tentacles but also part of the zoïd. Berninger ('10) found that, in response to inanition, hydra lost its tentacles. Finally Kepner and Jester ('27) also observed that the loss of tentacles was brought about in response to inanition. This loss, according to them, was accomplished by ingestion of the tips of the tentacles through the mouth. This may occur, but undoubtedly is not the usual method, as Hyman ('28) indicated.

It is a well known fact that the concentration of the hydrogen ion medium that bathes the protoplasm or protoplasmic tissue

¹ These investigations were carried on under the direction of Professor W. A. Kepner. Acknowledgments are due Mr. Carl H. McConnell of this laboratory, for the preparations of the photomicrographs.

has a profound effect upon it, therefore it seems strange that no attempts have been made to account for reduction, dedifferentiation and resorption on the basis of such environmental conditions. The following observations and results have been obtained through an effort to determine whether or not the concentration of the hydrogen ion is an important factor with reference to the three above mentioned phenomena.

METHODS AND MATERIALS.

Filtered spring water in 300 cc. portions kept in thoroughly cleansed glass dishes was used as a culture medium.

Very dilute solutions of $N/20$ sodium hydroxide and of hydrochloric acid were used in quantities to adjust the pH of the solutions. The colorimeter method was used for the pH determination of the solutions and LaMotte color standards were employed for color matching. Tests, adjustments and observations were made every twenty-four hours except where otherwise indicated. The temperature was maintained between 18 and 22° C. During these investigations frequent examinations were made of both the culture and of the animals for protozoa which might have been responsible for reduction. None were found except where stated. Observations were made with a dissecting binocular of a magnification of twenty diameters. These observations were supplemented by histological preparations.

At first distilled water was tried as a culture medium with the idea that a more accurate determination could be made of the hydrogen ion concentration. Various deleterious factors enter into the use of such a medium so it was discarded. In the subsequent experiments, filtered spring water was used.

The terms reduction, dedifferentiation and resorption, as used by other authors and us, may be defined as follows: Reduction is a uniform decrease in surface area in which process the ectoderm, mesoglea and endoderm remain intact and maintain a normal position in relation to each other. Dedifferentiation and resorption represent a dual phenomenon which involves a local reduction of surface. The presence of this dual phenomenon in the tentacles is indicated by a thickening and knobbed appearance at the tips of the tentacles.

EXPERIMENTAL.

Culture 1.—Four *Pelmatohydra oligactis* (Pallas), were placed in a culture medium consisting of distilled water and NaOH was added to maintain a constant pH of 7.8. At the end of a period of six days there was much apparent reduction and resorption of the tentacles in all specimens. One polyp was fed on the sixth day and one on the seventh. At this point the experiment was terminated through an accident.

Culture 2.—Four *Pelmatohydra oligactis* were placed in a culture medium of distilled water. The culture maintained a pH of 6.8 without the addition of either hydrogen or hydroxyl ions. These polyps disintegrated in five days.

Culture 3.—Four *Pelmatohydra oligactis* were placed in a culture medium consisting of distilled water. This culture maintained a pH of 7.0 which was fatal to the polyps in five days. At this phase of our observations we came to the conclusion that we were imposing other factors than the controlled pH represented, upon the hydras in using distilled water. A change in osmotic pressure was undoubtedly involved when distilled water was used instead of spring water. So, from this point on, spring water was employed as the medium in which to keep the observed polyps.

Culture 4.—Four *Chlorohydra viridissima* (Pallas) were taken from spring water which tested pH 7.6. They were normal in every respect. The pH of the second lot of spring water was now maintained at 6.6. The only change being made here was using a second glass dish similar to the one in which the pH tested 7.6 and in the pH now being 6.6. In five days, six of the polyps had disintegrated and the remaining one had undergone advanced dedifferentiation and resorption. It was placed in filtered spring water of pH 8.6 in an effort to bring about regeneration but it disintegrated in a few hours. This result, together with general observations made on various cultures, in the laboratory, in which the polyps displayed marked dedifferentiation and resorption, indicates that the acid condition of the medium induces dedifferentiation and resorption. Our observation upon a lower hydrogen ion concentration (higher pH) proved to be little more instructive as seen by the following culture.

Culture 5.—Six *Pelmatogydra oligactis* were isolated in filtered spring water the pH of which was maintained between 7.6 and 8.2. On the 8th day all of the hydras appeared perfectly normal; however, on the 9th day, all except one had disintegrated. The one remaining hydra showed no apparent reduction or de-differentiation and resorption of the tentacles. This hydra was sectioned and its histology appears later in the paper.

On several occasions similar results were obtained when the pH was held within the range from pH 7.8–8.0. It appears that the first ten days represent a critical period when the polyps are exposed to inanition. After the 10th day has passed we have had uniform results as the following observations indicate.

Culture 6.—Four *Chlorohydra viridissima*, in which some resorption was displayed, were isolated in filtered spring water pH 6.6. This water was over *Elodea* which had been previously boiled. The *Elodea* was separated from the polyps by a double thickness of cheese-cloth spread over the bottom of the container. The *Elodea* was removed after six days and spring water alone was used. As indicated above, these hydras were in a somewhat resorbed condition. The pH of this culture was varied, first decreasing the concentration of the hydrogen ions after the first two days up to 7.6, then increasing to 7.0, then again decreasing to 7.8. A pH of 7.8 was maintained for the last thirteen days. Immediately following these changes in pH, we observed the physiological aspect of the polyps. It was seen that the greater the concentration of the hydrogen ions the greater was the degree of dedifferentiation and resorption in the polyps. If the concentration of the hydrogen ions was lessened the hydras returned to normal. Two of the four hydras survived for a period of twenty-three days. One of these was sectioned (its histology is referred to later in the paper) and the other was lost during a transfer for examination. On the nineteenth day a green hydra, with much resorbed tentacles and bearing gonads, was introduced into this culture. In two days this hydra had gained its normal appearance but its gonads had partially disappeared. It was fed and placed in an aquarium containing food where it developed into a fine vegetative specimen apparently normal. In this last specimen the change from laboratory culture water to filtered spring water must have been a factor as well as the change in pH.

This does not however lessen the significance of the reaction of the other individuals of culture 6, wherein only the pH concentration has been the factor involved.

Culture 7.—Six *Chlorohydra viridissima* in a slightly resorbed condition were placed in filtered spring water without *Elodea* the pH of which tested 8.6. After the first two days the pH was maintained at 7.8 until this experiment was terminated. On the fourteenth day one hydra was sectioned. At the end of a period of twenty-four days three hydras remained. They were much reduced in size but their tentacles were apparently normal. On the twenty fifth day they were placed in an aquarium containing food where they lived for several days and attained nearly normal size. At this point our observations on these animals ceased.

These most interesting cases (cultures 6 and 7), in which the polyps that had been reduced and in which apparent dedifferentiation and resorption had taken place at a hydrogen ion concentration above the optimum, were restored to a completely normal condition when subjected to hydrogen ion concentration at or near the optimum. This undoubtedly indicates that food is not necessary for the regeneration of hydra, but regeneration depends rather upon the hydrogen ion concentration of the culture water. Kepner and Jester ('27) record one hydra which had lost all of its tentacles and without the presence of food the lost tentacles were replaced by regenerated ones in eight days. As the culture medium was frequently changed it is probable that a favorable pH was accidentally maintained. Hyman ('28) records the same phenomena when she says: "Depressed specimens may be caused to regenerate if the water is replaced by culture water" (page 78). Huxley and DeBeer in working with *Obelia* and *Campanularia* were unable to cause the regeneration of dedifferentiated and resorbed tissue.

Culture 8.—Eight *Pelmatohydra oligactis* were isolated in filtered spring water the pH of which was maintained for the first two days at 8.4 and for the remainder of the period it was kept at pH 7.8. On the tenth day three hydras had completely disintegrated without displaying reduction, dedifferentiation and resorption. On the 17th day, *Halteria* appeared in the culture. These were not abundant, about ten being found in the

field of the binocular dissecting microscope. As all the hydras appeared in the same condition one was sectioned. These sections showed no *Halteria* present within coelenteron or the food vacuoles. But menatocysts were present in the epitheliomuscular cells of the endoderm and within the coelenteron, hence the histology indicates that resorption had taken place. This resorption was so slight that it is overlooked by examination of the living polyps under a dissecting microscope. The culture medium was changed, so as to have water free of protozoa, and the observations continued. On the twenty third day one hydra was sectioned (its histology is referred to later). On the twenty fifth day the remaining three hydras were given bits of liver which they readily accepted. Thus indicating that they were not in a "depressed" condition as described by Hyman ('28). They were placed in an aquarium containing food where they were observed for several days. No indication of "depression" became evident during these observations nor was there any evidence of it at the time the observations ceased.

In order to determine wherein the optimum range of hydrogen ion concentration for the medium lay, both green and brown hydras were exposed to varying degree of hydrogen ion concentration ranging from pH 5.2-8.0 and the time recorded when all hydras had disappeared in each culture. The result of this experiment is given in the following table.

Four more cultures were run, with both green and brown hydras, one with a pH of 7.8, the other at pH 8.0. All the polyps in these cultures were alive at the end of a period of twenty four days.

This indicates that the optimum hydrogen ion concentration lies near pH 7.8. And further hydrogen ion concentration is an important factor in the determination of dedifferentiation and resorption; for, in the same medium (filtered spring water) with only the concentration of hydrogen and hydroxyl ions altered, we have been able to either induce or inhibit dedifferentiation and resorption. This does not support the later part of Hyman ('28) page 93, paragraph 2, Biological Bulletin volume LIV, January 1928, number 1 in her explanation of the phenomenon of depression when she says that "it is induced by transfer to clean fresh water." It is quite evident that, if two different lots of hydra

TABLE I.

THE X MARK INDICATES THE DAY OF THE DEATH OF THE LAST HYDRA IN THE CULTURE.

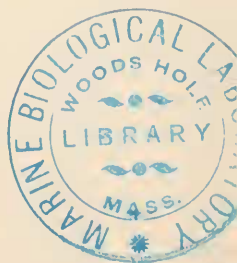
	Existence in Days.											
	2	3	4	5	6	7	8	9	10	11	12	13
Brown hydra in pH 5.2.....	×											
Green " " " 5.2.....		×										
Brown " " " 5.4.....		×										
Green " " " 5.4.....			×									
Brown " " " 5.6.....			×									
Green " " " 5.6.....			×									
Brown " " " 5.8.....			×									
Green " " " 5.8.....			×									
Brown " " " 6.0.....					×							
Green " " " 6.0.....					×							
Brown " " " 6.2.....					×							
Green " " " 6.2.....					×							
Brown " " " 6.4.....						×						
Green " " " 6.4.....						×						
Brown " " " 6.6.....						×						
Green " " " 6.6.....						×						
Brown " " " 6.8.....							×					
Green " " " 6.8.....							×					
Brown " " " 7.0.....							×					
Green " " " 7.0.....							×					
Brown " " " 7.2.....								×				
Green " " " 7.2.....							×					
Brown " " " 7.4.....									×			
Green " " " 7.4.....							×					
Brown " " " 7.6.....												×
Green " " " 7.6.....										×		

taken from the same culture or aquarium are placed in identical spring water cultures save for the concentration of the hydrogen ions and favorable reactions are repeatedly to be noted in the culture of low hydrogen ion concentration while unfavorable reactions are always to be noted in the culture of high hydrogen ion concentration, undoubtedly the pH of the culture must be a strong factor in determining this difference in the reactions

HISTOLOGY.

The histology of reduction, dedifferentiation and resorption in Hydra has been observed by E. Shultz ('06) and W. Rehm ('25). Huxley and DeBeer ('23) also described histologically dedifferentiation and resorption in *Obelia* and *Campanularia*. Our observations are almost in exact agreement with those of the above.

Studies on reduction, dedifferentiation and resorption always



involve the histology of the animal. Hydra is a diploblastic animal having only an ectoderm and endoderm. The ectoderm presents in its vegetative condition, epithelio-muscular, interstitial, cnidoblastic and nerve cells. The endoderm, on the other hand, is made up of epithelio-muscular, glandular, interstitial and scattered or isolated nerve cells. In the ectoderm there is no great local specialization or differentiation into regions. The ectoderm, however, shows three distinct regions: (1) the oral two-thirds in which there are scattered gland cells and a general covering of epithelio-muscular cells that are heavily charged with absorbed alimentary products; (2) a basal third that has few if any gland cells and in which the epithelio-muscular cells are usually highly vacuolated, except for those at the basal disc and (3) the endoderm of the tentacles. In this third region there are no gland cells and the epithelio-muscular cells are highly vacuolated. Thus it appears that the endoderm of the highly active or moving tentacles resembles that of the relatively quiet basal third of the body proper.

Dedifferentiation and resorption have been referred to frequently above. This has been defined as a dual phenomenon which involves a local reduction of surface. We take the presence of ectodermal elements (nematocysts being the most easily recognized) within the coelenteron or endoderm as evidence that dedifferentiation and resorption have taken place.

The question now remains: How is the surface reduced locally, and how do ectodermal elements gain their entrance into the coelenteron? As this phenomenon is most often seen in the tentacles, we have studied it there. In response to adverse environmental conditions, the cells at the tips of the polyp's tentacles coalesce or become dedifferentiated. The ectoderm is apparently affected first. Here the dedifferentiated cells, preparatory to resorption, group themselves into rounded or spheroidal masses. (Fig. 1-A.) Nematocysts as well as numerous cell-fragments may be seen within these aggregates. Obviously there must be some change in the non-living mesoglea as well as the living endoderm before resorption of the modified ectoderm can proceed. Dedifferentiation, therefore, starts in the endoderm. These cells, apparently, break away from the walls of the tentacles and soon assume a globular form (Fig. 1, B).

They migrate down the lumen of the tentacle (Fig. 1, B). Now the mesoglea breaks or is resorbed (Fig. 1, C) and the endodermal elements apparently have little trouble in finding their way to the coelenteron. The cellular masses of ectoderm, spheroidal in shape and often with contained nematocysts, together with the above mentioned dedifferentiated endo-epithelial masses, may be found in the coelenteron as far down as the basal disc. Thus the surface of the tentacle is decreased. To use the language of Huxley and DeBeer ('23) in describing a similar phenomenon in *Obelia* and *Campanularia*, "The ectodermal cells may be compared with that of a rear guard, retreating yet always maintaining an unbroken front." These histological details serve as a final criterion for determining whether dedifferentiation and resorption have taken place. But with the aid of low magnification, one can see that, as resorption proceeds, the tips of the tentacles increase in diameter, and finally appear knobbed and the involved area becomes darker and darker. The endodermal cells lining the tentacles are normally highly vacuolated. These cells, however, appropriate relatively much food during the later stages of resorption.

It is certain that this dedifferentiated and resorbed tissue is used as food by the animal because nematocysts in various stages of digestion may be found in the epithelio-muscular cells in all parts of the endoderm. This confirms Kepner and Jester ('23) in their minor claim that the ingested parts were used as food; but Kepner and Jester were misled by the occasional biting off of the tentacles. Dedifferentiation and resorption are the usual reaction.

Since it was seen that both the cells of the ectoderm and the endoderm of the tentacles were almost exactly like those of the lateral walls of the basal one third of hydra, dedifferentiation and resorption was looked for in this basal region. It was found to occur in the case of the sectioned hydra recorded in culture number 5 (Fig. 2, A). No explanation is offered for dedifferentiation and resorption being found in the basal disc in this and no other case. It was noticed, however, that in this case resorption was not found in the tentacles. Resorption has not been reported before as occurring in the basal region prior to its inception in the tentacles and peristome. All other writers state that it

starts at the tentacles and proceeds towards the base. The peristome is affected, according to them, after the tentacles have been removed. But this specimen showed dedifferentiation only in the basal region.

Green hydra reported in culture number 6 which was carried twenty-three days without food, showed histologically only slight resorption.

Rehm ('25) says that at the end of twenty one days the body of hydra subjected to inanition was reduced to a mere rounded form, which he calls, following Will and other investigators, "Reduktionskörper" (§ 371). At other places he refers to these rounded hydras as presenting planula-like pictures ("planula-ähnliches Gebilde, der Reduktionskörper") (§ 382). We have carried brown hydra for twenty three days within the optimum hydrogen ion concentration. This polyp showed so little dedifferentiation and resorption that they could only be detected histologically. Under low magnification the living polyp, though reduced in size, appeared to be complete and have no broken surface. The brown hydra, as recorded in culture number 8, which was sectioned after sixteen days of inanition within the optimum hydrogen ion concentration, presented, while living, no evidence of dedifferentiation and resorption under low magnification. However, the histology of this animal shows frequent nematocysts in the coelenteron hence slight dedifferentiation and resorption must have taken place during the seventeen days of inanition. Examination on this day under the dissecting microscope disclosed no difference in appearance between the remaining hydras and the one sectioned. On the twenty third day another hydra from this culture was sectioned. From the histology of this polyp, it is seen that dedifferentiation and resorption which were shown in the histological examination of the hydra sectioned on the 17th day not only has ceased but the resorbed tissue has been digested by the polyp sectioned after twenty three days of inanition within the optimum range of hydrogen ion concentration. Similar phenomena have been observed for green hydras. For a green hydra, which had suffered 14 days of inanition at optimum hydrogen ion concentration showed slight dedifferentiation and resorption; while a second green polyp, from the same culture sectioned after twenty three days of inanition at optimum hydro-

gen ion concentration, showed no evidence of dedifferentiation and resorption.

Thus it appears that during inanition at optimum hydrogen ion concentration a crisis is reached after about two weeks. During this crisis slight dedifferentiation and resorption make their appearance. The resorbed material may supply sufficient nourishment to tide the polyp, now reduced in size, through a long period before a second crisis develops and compels the dedifferentiation and resorption of more tissue.

SUMMARY.

1. The optimum range of hydrogen ion concentration for both *Hydra viridissima* and *Pelmatohydra oligactis* lies within the range pH 7.8 and 8.0.
2. Polyps allowed to develop pronounced dedifferentiation and resorption in a high hydrogen ion concentration (low pH) were induced to completely restore their lost parts when the medium was altered to be within the optimum range of pH.
3. Hydras carried within the optimum range of pH were subjected to periods of inanition as great as twenty five days without showing any external evidence of dedifferentiation and resorption at the end of this period.
4. Histological preparation of polyps, kept for long periods without food at the optimum hydrogen ion concentration, show slight evidence histologically of dedifferentiation and resorption at a critical period. This critical period appears somewhere between ten and seventeen days after inanition within the optimum range of pH. Such microscopic dedifferentiation and resorption are not progressive; for after this critical period has passed no further histological evidence of dedifferentiation and resorption has been observed.
 - (b) This microscopic dedifferentiation and resorption usually appear at the tips of the tentacles; but in one case we have seen it involve the basal third of the polyp and not the tentacles.
5. Hydras subjected to long periods of inanition within the optimum range of pH accept food readily. There is, therefore, no evidence of depression given by these polyps.
6. Dedifferentiation and resorption are induced rather by unfavorable hydrogen ion concentration than by inanition.

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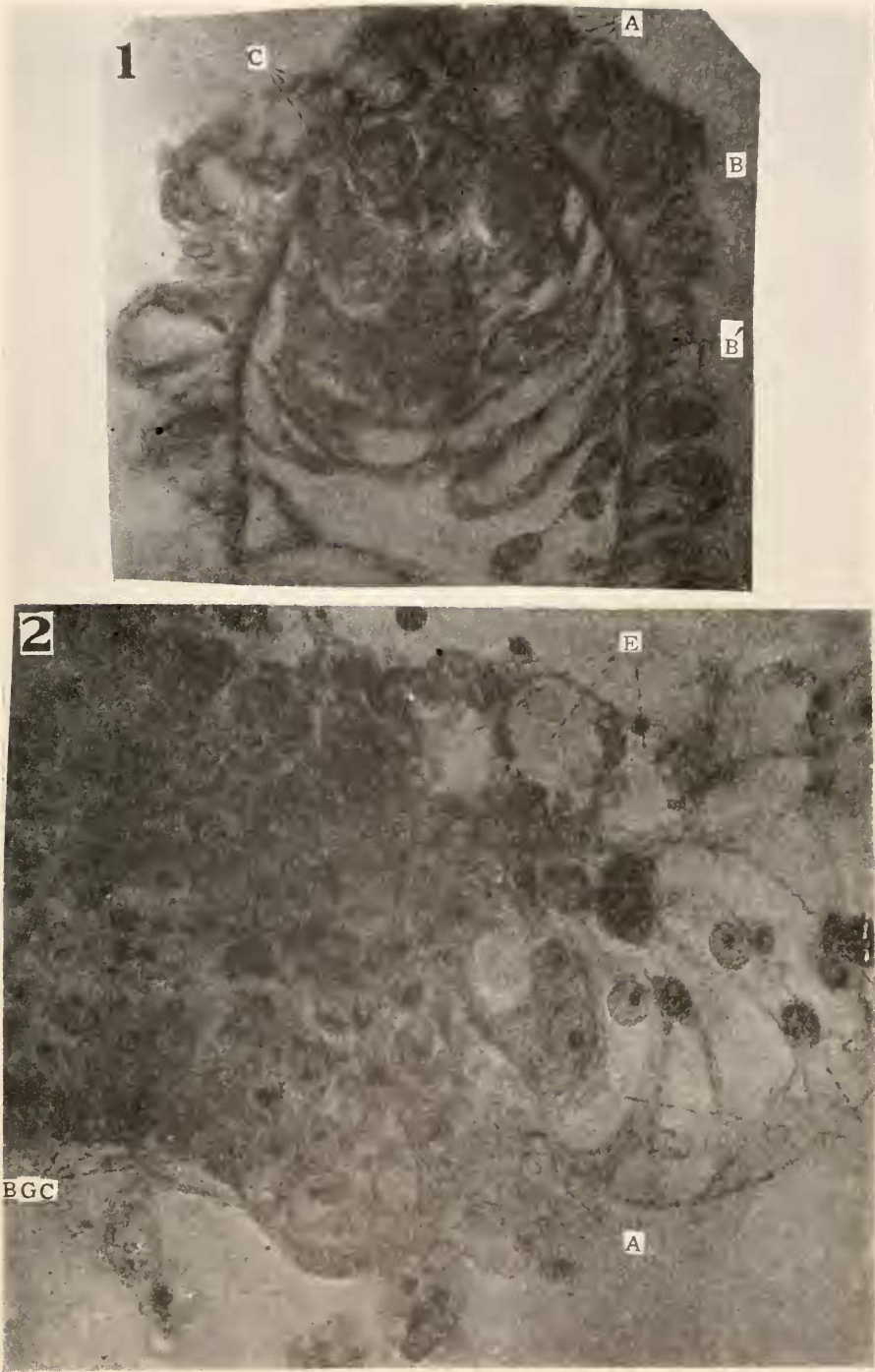
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PLATE I.

Explanation of Figures.

FIG. 1. Longitudinal section of the free end of a tentacle of *Pelmatohydra oligactis* which had been starved twenty-four hours in spring water at pH 6.8. This shows the inception of dedifferentiation and resorption. The mesoglea has broken down at end of tentacle. Rounded masses of coalesced ectodermal cells are forming (A). Similar rounded masses of coalesced endodermal cells are forming (B); at B' we see a mass of coalesced endodermal cells having migrated towards the lumen of the tentacle; at C a mass of coalesced ectodermal cells is passing through the region of the broken down mesoglea. $\times 700$.

FIG. 2. A longitudinal section involving a part of the basal disc of *Pelmatohydra oligactis*. (Culture number 5.) This specimen had been starved nine days within optimum hydrogen ion concentration. The inception of dedifferentiation and resorption is shown at A; BGC, basal disc glands cells; E, endodermal cells; L, lateral ectodermal cells. $\times 700$.



THE OCCURRENCE OF NUCLEAR VARIATIONS IN *PLEUROTTRICHA LANCEOLATA* (STEIN).

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The occurrence of variations from the accepted type among the protozoa has received much attention in recent years, and a number of such cases have been reported, both of the artificially induced and spontaneously appearing sort. Most of the former have been of the "enduring modification" type, that is they persist throughout a longer or shorter period of vegetative division, but are eventually lost when conjugation or endomixis takes place. The latter may be divided into two classes. The first group would include the true mutations, of which the tetraploid *Chilodon* described by MacDougall (1925) is probably one of the best authenticated examples. In this case the mutation, which consisted in the possession of twice the usual number of chromosomes, combined with unusual size and certain other minor characteristics, persisted through both conjugation and division. To the second group would belong all other departures from normal, such as the production of monsters, the amiconucleate condition in infusoria, and various other unusual physiological and morphological characters which persist through division but tend to revert to normality eventually. Examples of this kind of variation are quite numerous. Among them may be mentioned the amiconucleate *Oxytricha* studied by Dawson (1919), the race of *Paramecium* which possessed extra contractile vacuoles (Hance, 1917), the rapidly-dividing race of *Didinium* reported by Mast (1917), and the sudden appearance of an *Arcella* having double characteristics described by Reynolds (1923). Since the latter investigator found that these abnormal characteristics could be diminished until a completely normal condition was reestablished, or increased by selection of suitable

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individuals this last variation evidently belongs with those found by Jennings (1920) and Root (1918) to exist in *Diffugia* and *Centropyxis*, with this difference, however—the former occurred suddenly, while the latter were of lesser degree and appeared more gradually. More recently Dawson (1924) has reported the occurrence of a peculiar form of *Paramecium aurelia* which has been carried in culture for several years since. The abnormal character in this case consists of a “notched” condition which is definitely heritable, at least in ordinary asexual division.

The present paper deals with variations in the number of both micro- and macronuclei in *Pleurotricha lanceolata*. Pedigreed cultures of this ciliate, which is a hypotrich belonging to the family Oxytrichidæ, were maintained for 18 months and studied mainly from the standpoint of the cytological changes occurring during conjugation and division, as described in a previous paper (Manwell, 1928).

The normal animal is shown in Fig. 1. It will be noted that it possesses two nuclei of each sort, and according to Stein (1858) who first described both the species and genus, the presence of two macro- and two micronuclei is a generic character. About two months before the culture was discontinued however, and while to all appearances it was in a very vigorous condition with division taking place very actively, individuals possessing only one macronucleus were noticed in some of the stained preparations. The micronuclear condition varied; in some cases there was only one and in others there were two as in normal individuals. Animals possessing the normal macronuclear complex but with three micronuclei have also been observed, and such changes are indeed not very uncommon, not only in *Pleurotricha* but in *Oxytricha* and other ciliates containing more than one micronucleus. But no individuals have been observed with only one macronucleus and more than two micronuclei. Fig. 2 shows an individual possessing but one nucleus of each sort in division, and in Fig. 3 a similar individual, differing only in having two micronuclei, may also be seen dividing. The next two figures show later stages in the division of such individuals, and in Fig. 6 a unimacro- and micronucleate animal is shown just after division.

From these figures it can be seen that division takes place in exactly the same way as it does in individuals having the normal

nuclear complex, and that the variations are heritable, at least in ordinary vegetative fission. To settle this point still more definitely several lines were started from individuals possessing but one nucleus of each sort and followed for 10 days. At the end of that time these subcultures were lost by accident and other circumstances made it necessary to conclude the experiment, but stained preparations made from each generation showed clearly that the reduced number of nuclei was being passed from one generation to the next.

A careful examination of stained preparations has been made in an effort to discover whether the abnormal nuclear complex was accompanied by any other morphological changes, but apparently there were none. During the early stages of division however (about the stage shown in Fig. 2) it was frequently possible to distinguish animals possessing but one macronucleus from normal individuals in the same culture in a similar stage, for the bodies of the former were definitely broader about $1/3$ of the way back from the anterior end and then tended to become narrower, while in the normal animals the entire middle third of the body was of a fairly uniform width. If there were any differences in size they were in favor of those individuals possessing but one nucleus of each sort.

No evidences of conjugation among these abnormal individuals was ever observed, but since as previously reported, conjugation occurred but rarely in all the cultures from start to finish of the experiment, not much stress can be laid on this point. Encystment was also not observed. Consequently it cannot be said whether such a variation as this would survive endomixis and conjugation, although it seems probable that in some cases at least, unimicro- and macronucleate conjugants might produce similar individuals.

In view of the work of Baitsell (1914), and the fact that conjugation in this species has been shown to result, at least when it occurs under cultural conditions favorable to vegetative division, in almost 100 per cent. mortality (Manwell, 1928) the question of the occurrence of such morphological variations as herein described becomes of some practical importance. For obviously, if under favorable conditions multiplication by fission can continue indefinitely, then such changes might be perpetuated for a very

long time in nature, as well as in artificial cultures. And if this is so account should be taken of the fact in the description of genus and species, since the number of nuclei, especially of the macronuclei, is a conspicuous character. If asexual reproduction can continue indefinitely then the sudden appearance of changes of the kind described would, for practical purposes, have the value of a mutation.

The occurrence of abnormal micronuclear conditions has been reported a number of times before, particularly with respect to the total absence of a micronucleus, and the presence of one or two supernumerary micronuclei is not very uncommon in species ordinarily possessing two or more, as already noted, but apparently the number of macronuclei is a much more constant character. The only instance in which a variation in the latter has been reported, to the author's knowledge, at least, is that given by Calkins (1926). Here he states (p. 579) that in early cultures of *Uroleptus mobilis* the number of macronuclei was almost uniformly 8, but as the age of the cultures increased individuals with a greater number of nuclei became common, until finally the number was nearly always 14 or 15.

SUMMARY AND CONCLUSIONS.

In a pedigreed culture of *Pleurotricha lanceolata*, a species of hypotrich normally possessing two macro- and two micronuclei individuals with only one macronucleus and one or two micronuclei suddenly appeared, at a time when division was rapid and the culture apparently very vigorous.

That the difference in nuclear number was heritable, at least in asexual multiplication, was shown from stained preparations and pedigreed lines, and the fact that it has been shown that this species will live and divide normally apparently indefinitely under favorable conditions, without conjugation, makes it probable that such variations as have been described would continue for a very long time, and that animals with such peculiarities may be common in nature as distinct varieties.

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EXPLANATION OF THE FIGURES.

Magnification $\times 550$; all drawings made with camera lucida

PLATE I.

FIG. 1. A typical vegetative individual.

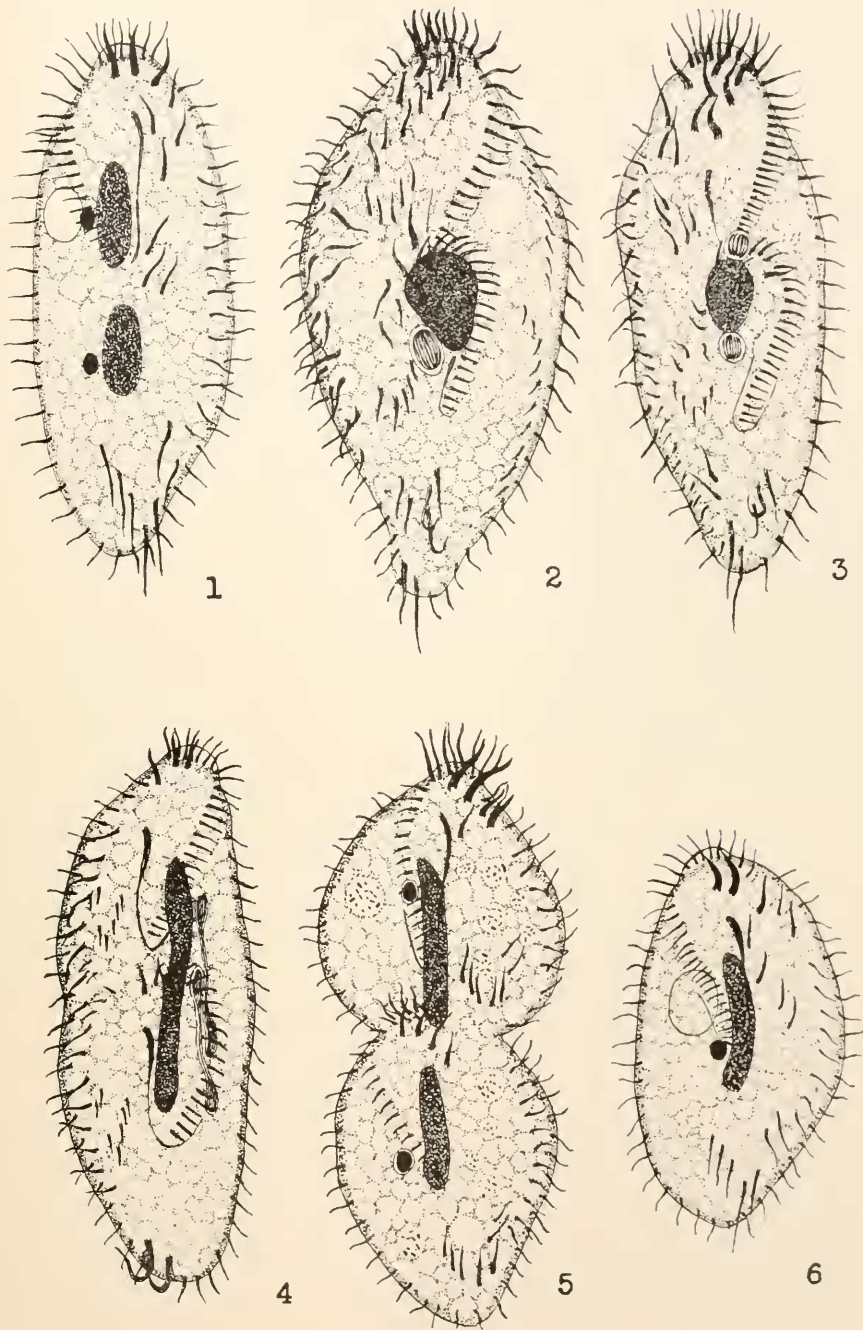
FIG. 2. An individual with one macronucleus and one micronucleus in a moderately early stage of division.

FIG. 3. A division stage similar to the above in an animal having two micronuclei, but only one macronucleus.

FIG. 4. A more advanced stage in an individual similar to the above.

FIG. 5. The final stage of division in a unimicro- and macro-nucleate individual.

FIG. 6. A daughter individual just after fission.



OBSERVATIONS ON THE LIFE HISTORY AND
PHYSIOLOGICAL CONDITION OF THE
PACIFIC DOG FISH
(*SQUALUS SUCKLII*).

J. P. QUIGLEY.¹

Incidental to an investigation of the reactions of *Squalus sucklii* to variations in the salinity of the surrounding medium (1) observations were made regarding the life history and physiological condition of this fish.

The fish were captured during the months of June, July and August of 1926 from the Straits of Georgia in the vicinity of Departure Bay, Vancouver Island, B. C. They were taken on a set line, the hooks of which were baited with pieces of salted herring. Most of the fish were obtained at a depth of about 30 meters, and they were generally caught near kelp beds. A sample of water taken at a depth of 30 meters in the region where many of the fish were taken was found by Lucas (2) to have the following characteristics; pH 8.4, temperature 10.3° C., density 1.0218, oxygen content 4.41 cc. per liter, sodium chloride content 27.37 gm. per liter.

Weight of Fish.—It was found that many of the factors associated with the weight of the fish could be emphasized by grouping the fish according to weight as has been done in Table I. Examination of this table shows that with the fish of lighter weight the two sexes are nearly equally represented, the number of males being slightly greater. As heavier fish are considered, the relative number of males shows a marked increase, then a sudden decrease so that in the weight divisions above 4,000 grams the males are entirely absent.

These results probably indicate that male fish with body weight over 4,000 grams do not exist in this locality during the summer. It cannot be definitely stated that the figures obtained with fish of lighter weight indicate the relative proportion in which the

¹ From The Pacific Biological Station, Nanaimo, B. C., and The Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta.

TABLE I.

Weight Limits (Grams).	Number of Fish Obtained.	Number of		Percentage.		Average Length (Cm.).	Average Increase in Length.
		Males.	Females.	Males.	Females.		
300-399...	12	7	5	58	42	39.9	
400-499...	15	9	6	60	40	43.6	3.8
500-599...	16	11	5	69	31	45.7	2.1
600-699...	13	8	5	62	38	48.4	2.7
700-799...	5	5	0	100	0	52.5	4.1
800-899...	7	4	3	57	43	53.8	1.3
900-999...	5	5	0	100	0	54.7	0.9
1,000-1,499	22	20	2	91	9	60.3	5.6
1,500-1,999	11	9	2	82	18	69.2	8.9
2,000-2,999	30	26	4	87	13	74.9	5.7
3,000-3,999	13	5	8	38	62	83.3	8.4
4,000-4,999	16	0	16	0	100	90.5	6.2
5,000-5,999	16	0	16	0	100	91.6	1.1
6,000-6,999	4	0	4	0	100	95.5	3.9
7,000-7,999	1	0	1	0	100	99.0	3.5

two sexes occur, although such probably is the case. Since the fish were taken on a set line hunger or greed might conceivably be a factor in determining whether or not fish would take the bait. The stomach of fish captured usually contained much food, a fact which indicates that feeding for this fish is determined more by the availability of food than by hunger.

Out of 219 fish captured, 128 (58 per cent.) were males. Craigie (3) examined the fish obtained in the same region during July and August, 1925, and found that among 76 specimens 44 (60 per cent.) were males, while during December of 1925 by examining 117 specimens he found 47 (40 per cent.) males.

As was to have been expected, there is a comparatively definite relationship between weight and length of fish. The increase in length is rather steady though not entirely uniform as heavier fish are compared with those of lighter weight. It could not be shown that sex altered the relation of weight and length. There was a slight though inconstant indication that nonpregnant females were longer than pregnant females of the same weight. The longest fish captured measured 99 cm., the shortest 35.5. The heaviest fish weighed 7,550 grams and the lightest 300 grams. When increasing their weight 100 grams the smaller fish made an increase in length of approximately the same magnitude as did the larger fish when making a weight increase of 1,000 grams.

Pregnancy and Embryos.—Of the females captured, 43 per cent. carried embryos large enough to be readily noted in a cursory inspection. The lightest fish having embryos weighed 3.440 grams and was 85 cm. in length. These figures give an approximate minimum limit of the size of the mature female. Among the 50 females captured with a weight equal to or above 3.440 grams, 39 (78 per cent.) carried embryos.

Ford (4) quotes the conclusion of several investigators that *Squalus acanthias* breeds throughout the year and of other investigators that this species breeds only during certain periods. The results of his own investigations support the latter conclusion and tend to show that near Plymouth, England, specimens ready for birth would not be found earlier than the end of August. I found specimens of *Squalus sucklii* embryos at all times during the summer which ranged through all the sizes from the smallest to those with the umbilical scar healed completely and apparently ready for birth. This observation naturally suggests that in the vicinity of Nanaimo, *Squalus sucklii* breeds at all times of the year.

In any one parent, the embryos were of the same general size. A set of developing eggs was always found in females carrying embryos. The number of embryos obtained from 16 fish varied between 3 and 11 with an average number of 6.87. Although it could not be definitely stated that none of the embryos had been lost from the mother in the course of capture it is believed that this was a rare occurrence. No embryos were lost after the mother was taken from the set line and in most cases egg capsules still unruptured were obtained. In an examination of *Squalus acanthias* Ford (4) found that females of this species could carry as many as 11 embryos but the greatest number of pregnant fish carried only 3. In *Squalus sucklii* I found that embryos of both sexes usually occurred in the same uterus but there was no relation between the number of either sex, e.g. in one fish I found 6 females and 1 male, in another 3 males and no females. Of the embryos obtained 50 per cent. were males. This figure is to be contrasted with that previously noted for the fish of small size taken on the set line where a preponderance of males existed. A blue shark, *Prionace glance*, (identified by Professor J. R. Dymond) received at the Pacific Biological Station, August 19,

1926, was found to have 11 females and 8 male embryos all the same size nearly ready for birth.

Constitution of Shoals.—Throughout the period fish were being taken, the specimens obtained on any set line usually consisted of both sexes in approximately equal numbers and of all sizes. The conclusion was reached that the shoals consisted of both sexes and all sizes of fish or else the line had been visited within a few hours by several different shoals. It was also noted that the largest fish were usually taken at a greater depth (very near or actually on the sea bottom) than the smallest and it may be that the composition of shoals is in part determined by size. From his study of *Squalus acanthias*, Ford (4) concluded that for this species the mature males and females each form separate shoals while these shoals in turn are distinct from those composed of immature males and females together. I obtained fish in the same region throughout the summer. It is therefore likely that certain shoals inhabit this region during the entire season.

SUMMARY.

1. Among the smaller fish males were slightly more prevalent than females. Males weighing more than 4,000 grams were not obtained. Females attain a much greater length and weight than males. The greater weight of the females was not always due to the presence of eggs or embryos.

2. A comparatively definite relationship exists between weight and length of fish. The relationship of length increase to weight increase for small fish is approximately ten times as great as for large specimens.

3. Of the mature females captured 78 per cent. carried embryos. This species apparently breeds throughout the year. The average number of embryos carried by the females is greater than six.

4. The shoals apparently consist of fish of all sizes and of both sexes. The shoals probably remain in the same region throughout the summer.

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ALGÆ OF PONDS AS DETERMINED BY AN EXAMINATION OF THE INTESTINAL CONTENTS OF TADPOLES.

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INTRODUCTION.

During the last few years a considerable amount of research has centered around the food taking of small fresh-water fish. This work has emphasized the dependence of small fish on algæ and in turn these fish as a source of food for the game fish. In reviewing literature the writer has found comparatively little scientific work on the feeding habits of the tadpole and frog.

The tadpole as well as the small fish is an indirect source of food for the human race. Tiffany ('22) states: "For most of the young fishes examined the complete story reads: 'no phytoplankton, no gizzard shad.'" It may also be said, no algæ, no tadpole.

The writer wishes to express her gratitude to Dr. Bruce D. Reynolds, who suggested this problem and who has greatly assisted by his advice and criticism in the preparation of this paper; also to Professor I. F. Lewis and Dr. E. M. Betts for helpful criticisms.

METHODS.

During the summers of 1927 and 1928 one hundred tadpoles and one hundred pond collections were taken from five ponds on the campus of the University of Virginia and in the surrounding vicinity. Two of the ponds measured approximately 250 ft. x 100 ft., one 150 ft. x 50 ft., one 100 ft. x 30 ft., and one 50 ft. x 20 ft. The ponds which were studied did not have active outlets.

Two examinations of each of these ponds were made during the summer of 1927 from July 15 to August 28, and two were made during the summer of 1928 from June 20 to July 5. Each collection from a pond consisted of five tadpoles¹ which measured from

¹ Of the 100 tadpoles used in these experiments, 94 were *Rana clamitans* and 6 *R. catesbeiana*.

one and three-fourths inches to five inches long and five collections of sediment taken from the edges of the ponds. The tadpoles and pond collections were put in separate containers. Immediately after returning to the laboratory the tadpoles were killed and the intestines removed. Three slides were made of material taken from each digestive tract, one from the anterior and one from the middle regions of the small intestine, the third from the anterior region of the large intestine. A study of each of the slides was made under the high power of the microscope. The algæ from each region were identified and recorded. The pond collections were studied in a similar way. Three slides were made from each of the pond collections. The algæ from each slide were identified and recorded.

During the summer of '27 the tadpoles were collected from the pond, and then the pond collections were made without any effort to correlate the position of the tadpole and the pond collection, but in the collections made during the summer of '28 a tadpole was caught and from the same place a pond collection was made.

THE PROBLEM.

The experiments presented in this paper were not undertaken primarily for the purpose of studying the food of tadpoles, but rather in order to ascertain if the algæ found in the alimentary tract of tadpoles can be relied upon as an index to the microscopic flora of the ponds in which the tadpoles are living. In other words, does the tadpole feed on different kinds of algæ or is it selective in its feeding habits? If not selective, is it as good a collector of algæ as the investigator interested in studying them?

EXPERIMENTAL.

In following up this problem observations were made on four collections, made at different times, from each of five ponds. The results obtained are shown in tabular form.

By referring to Table I. it will be seen that the number of species of algæ obtained from the intestine of the tadpoles exceeded the number obtained from the pond collections in every case except two, and in these instances they were the same—the pond collections being made where the tadpoles were caught.

Attention is also called to the relative number of algæ found in

the intestines of tadpoles and the ponds from which they were taken, in large and small ponds (Table I.). It is evident that, when making collections from small ponds, the investigator is able to find most of the algæ present; whereas if the pond is a large one there is an appreciable difference between the number of species of algæ obtained by the two methods—the ratio being approximately 4:3 in favor of the tadpole.

TABLE I.

SHOWING THE TOTAL NUMBER OF SPECIES OF ALGÆ TAKEN FROM THE INTESTINAL TRACT OF FIVE TADPOLES AS COMPARED WITH THE TOTAL NUMBER FOUND IN FIVE COLLECTIONS MADE FROM THE SAME PONDS.

Size of Pond.	Collections Made during Summer of 1927.				Collections Made during Summer of 1928.			
	Jun. 15–Aug. 11.		Aug. 11–Aug. 28.		Jun. 21–Jun. 27.		Jun. 27–July 5.	
	Tadpole.	Pond.	Tadpole.	Pond.	Tadpole.	Pond.	Tadpole.	Pond.
250 x 100 ft.	50	32	59	39	63	49	58	48
250 x 100 ft.	54	42	45	37	44	44	56	49
150 x 50 ft.	52	46	47	44	65	56	59	46
100 x 50 ft.	35	30	63	50	56	47	47	41
50 x 20 ft.	35	30	46	44	47	39	44	44

As stated in a paragraph under Methods, three examinations were made of each pond collection and of each tadpole—one from the anterior region of the small intestine, one from the middle region of the small intestine, and one from the large intestine. Table II. shows the distribution of the species in different regions of the intestinal tract as compared with the total number found in the tadpole and the total number found in the pond collections. Usually more species of algæ were found in the anterior end of the small intestine, but there is not a great variation in numbers in the three regions. Most of the algæ found in the large intestine show slight evidence of having been acted upon by the digestive juices.

Even though the species of algæ found in the tadpoles outnumbered those in the pond collections, algæ which did not occur in the tadpoles' intestines were found in collections made from the pond. There was one exception, and in this case the tadpole and pond collection were taken from the same place. In this entire work only five species of algæ were found in pond collections

TABLE II.

SHOWING THE TOTAL NUMBER OF SPECIES OF ALGÆ FOUND IN DIFFERENT PONDS,
THE NUMBER FOUND IN TADPOLES AND THE NUMBER FOUND IN
DIFFERENT REGIONS OF THE INTESTINE.

A. S. Int., anterior end of small intestine; M. S. Int., middle region of small intestine; A. L. Int., anterior end of large intestine.

Pond.	Tadpole.	A. S. Int.	M. S. Int.	A. L. Int.
29	36	23	22	20
30	34	22	16	19
35	44	23	24	19
33	33	24	19	19
30	45	21	24	31
28	33	28	26	21
31	40	26	20	24
32	31	19	14	14
27	50	26	21	36
16	34	18	13	16
25	35	29	12	16
18	35	26	14	22
35	34	21	26	16
24	36	21	21	27
32	38	21	16	21
27	36	20	22	22
32	34	31	15	18
25	38	22	21	25
24	32	18	18	19
36	49	32	26	30

TABLE III.

COLLECTIONS MADE DURING SUMMER OF 1927.

Total Number Species from Both Sources.	Percentage of Those Found in Tadpoles.	Percentage of Those Found in Pond.	Total Number Species from Both Sources.	Percentage of Those Found in Tadpoles.	Percentage of Those Found in Pond.
50	86.20	55.17	70	82.85	55.71
68	79.32	61.76	58	83.10	63.79
70	74.28	65.71	57	82.62	77.19
45	77.77	66.66	68	93.64	73.23
37	94.59	81.08	53	86.79	75.28

COLLECTIONS MADE DURING SUMMER OF 1928.

67	94.03	73.13	64	95.31	77.50
50	88.	88.	58	96.55	84.48
66	98.48	84.84	62	95.17	74.19
60	94.33	78.33	56	100.	83.91
48	97.91	81.25	47	93.61	93.61

Showing total number of species of algæ taken from each pond, including the percentage of those obtained from tadpoles and from pond collections.

which were not also observed in the tadpoles. Evidently these species were very rare, for only one was encountered the second time. The fact that these algæ were not found in the tadpoles does not indicate, therefore, that the tadpoles refuse to eat them.

The variation in percentage of algæ from the two sources is less when pond collections and tadpoles are taken from the same place. This may be seen by referring to Table III. The pond collections made during the summer of 1928 were taken from the immediate vicinity in which the tadpoles were caught, while those made during the summer of 1927 were taken without regard to this matter.

SUMMARY.

It is a well known fact that tadpoles feed on microscopic plants. The importance of this animal as a collector of algæ is clearly demonstrated. In comparing the intestinal contents of one hundred tadpoles with pond collections made from the same ponds, the number of species of algæ obtained from the tadpoles exceeded the number obtained from the collections in every case except two; and in these instances, they were the same. It may be stated, therefore, that an examination of the intestinal contents of tadpoles affords one of the best and easiest methods of determining the species of algæ present in ponds. This is especially true in large ponds, and applies particularly to the phytoplankton.

In this examination one hundred and seventy species and varieties of phytoplankton were found. Of this number, one hundred and sixty-five were encountered in the intestines of tadpoles.

CONCLUSION.

1. The food of green-frog tadpoles consists chiefly of algæ.
2. The algæ from pond collections and from the intestinal contents of tadpoles taken from the same ponds do not differ as much in small ponds as they do in the larger ones.
3. The anterior region of the small intestine is considered to be the best region for making examinations for algæ.
4. The species of algæ taken from the intestines of tadpoles constituted, on the average, $89.73 \pm$ per cent. of the total found.

5. An examination of the intestinal contents of tadpoles affords one of the best and easiest methods of obtaining a collection of algae from ponds.

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FURTHER OBSERVATIONS ON THE CHEMICAL
COMPOSITION OF WOODS HOLE SEA
WATER—THE CHLORINE
CONTENT AND SALT
ANALYSIS.

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From time to time we have had occasion to make further observations on the sea water at Woods Hole since the publication of the original analysis (1). Though not in any sense complete it is believed that the following data may prove useful and therefore they are presented.

It should be pointed out that our aim has been always to select methods of analysis which would adapt themselves to the use of relatively small fluid volumes, as only in this way can they become applicable to the investigation of physiological and biological problems. From the large number of analyses of sea water tabulated by the Hydrographic Laboratory of Copenhagen, Knudsen, Dittmar (2) etc., further data of this kind have oceanographic interest but little more. There has, therefore, been made a conscious attempt to utilize more sensitive methods which require small samples for analysis, albeit the absolute values *may* not be quite as accurate.

DETERMINATION OF CHLORINE.

Since many physiological activities are sensitive to slight changes in the tonicity of the surrounding medium it seemed of interest to determine whether the chlorine content of the Woods Hole sea water varied to a significant degree from day to day. The method employed was as follows: Standard AgNO_3 was made such that 1 cc. was equivalent to 10 mg. chlorine. This was standardized against pure NaCl since it has been shown by Thompson (3) that this salt may be substituted for standard water from the Hydrographic Laboratory. The AgNO_3 was

kept in the dark in a glass stoppered brown bottle and the standardization repeated at the end of the series of determinations. The method, thereafter, followed in detail that presented by the Association of Official Agricultural Chemists (4). The burette used was of 50 cc. capacity, standardized by the Bureau of Standards, Washington. 15 cc. samples of sea water were measured with a standardized pipette and diluted with distilled water to 35 cc. before titration.

Samples were taken from the laboratory tank. This tank is fed by water taken about 125 feet from shore. The other samples were taken from surface water as follows: (1) Buzzards Bay one half mile North of Robinson's Hole. (2) Cuttyhunk 300 feet from shore on the "Sound" side. (3) Tarpaulin cove one half mile out in the Sound; water 80 feet deep. (4) East of Nobska; water 28 feet deep.

Duplicate titrations were made and it may be said that these determinations but rarely disagreed.

The temperature was taken with not great accuracy, employing a standard 50 degree laboratory thermometer. Such slight changes as observed during these observations were not considered significant.

Grams of chlorine per kilogram were calculated from Thompson's empirical formula—

$$Cl_w = 0.008 + 0.99980 Cl_v - 0.001228 Cl_v^2$$

where Cl_w = grams of Cl per kilogram and Cl_v = grams Cl per liter at 20° C. A graph prepared by using the more common range of Cl contents was found useful.

The salinity—defined as the weight in grams of all the salts dissolved in a kilogram of sea water, after the carbonates have been converted to oxides, the Br and I have been replaced by Cl and the organic matter has been completely oxidized—was calculated from the relation derived by Knudson—

$$So/oo = 0.030 + 1.8050 Cl_w$$

Of course it must be recognized that this is only an approximation, as Giral (5) has emphasized.

During these observations it should be stated that the weather was in general extremely bad, rain alternating with fog for dis-

TABLE I.

CHLORINE CONTENT OF WOODS HOLE SEA WATER DURING THE SUMMER OF 1928.

Date.	Source.	Temperature.	Grams Cl per Liter.	Grams Cl per Kilogram.	So 'oo.
July 16...	Laboratory tank	21 degrees	17.80	17.42	31.47
" 18...	" "	22 "	17.80	17.42	31.47
" 21...	" "	21 "	17.86	17.48	31.58
" 23...	" "	21 "	17.77	17.39	31.42
" 26...	" "	21.8 "	17.77	17.39	31.42
" 28...	" "	21 "	17.86	17.48	31.58
August 1	" "	20.5 "	17.80	17.42	31.47
July 17...	Buzzards Bay...	20 "	17.93	17.54	31.69
" 17...	Cuttyhunk	20 "	18.00	17.60	31.79
" 21...	Off Tarpaulin Cove	20 "	17.93	17.54	31.69
" 21...	East Nobska	20 "	17.70	17.32	31.27

agreeably long intervals. The results, do not show any very marked changes in the Cl content of the water but it is altogether possible that a dry summer may increase the Cl content. Samples taken from other points along the uneven coast of Woods Hole show more evident variations, as was to be expected.

SEA SALT ANALYSIS.

Samples of the dried sea salt taken from the laboratory tank during the summer of 1926 have been analysed, employing the classical methods as given in the Bulletin of the Official Agricultural Chemists (4) and by Scott (6). Though not complete, these data are presented, as they may be found useful.

SEA SALT OF WOODS HOLE.

	Percentage.	
	No. 1	No. 2.
Sodium.....	30.68	30.49
Magnesium.....	3.31	3.48
Calcium.....	1.27	1.12
Silica.....	0.014	0.018
Phosphate.....	Trace	Trace
Nitrate.....	Trace	Trace

The above analyses would tend to confirm the suggestion made in our former paper that the Kramer-Gittleman direct method for the determination of sodium, while very convenient for relative data, may give an absolute value which is low. One must remember, however, that using the Haywood and Smith Method

(7) or that of Dittmar the sodium determination comes out low, as has been the universal experience of analysts. The values are then corrected by employing Dittmar's method (2) of "total sulphates." The older methods for sodium determinations are so cumbersome (as reference to Dittmar's article will show) that there is still some doubt as to the accuracy of the results.

During the Summer of 1928 we have again confirmed Atkins' (8) and Harvey's (9) work on the nitrates and phosphates. Samples of the Woods Hole water showed only the smallest trace of NO_3 and PO_4 during July 1928, the time at which our analyses were made this year. This change is, as they have shown, due to seasonal variations in the plankton.

SUMMARY.

1. The chlorine content of Woods Hole sea water has been examined over a three-week period and shown not to vary within any large range.
2. Analyses of the sea salt are presented.

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THE PRECIPITATION OF CALCIUM AND MAGNESIUM FROM SEA WATER BY SODIUM HYDROXIDE.

ELEANOR M. KAPP.¹

In the course of an investigation into the modification of sea water for use as a perfusion medium (Kapp, '28), it became necessary to know something of the relative amounts of calcium and magnesium precipitated by sodium hydroxide. Haas ('16) suggested that the first flat portion of his titration curve for sea water was coincident with the precipitation of Mg as hydroxide, the second with that of Ca. That this was a reasonable assumption is further suggested by the solubility product constants for the hydroxides of Mg and Ca, which are 1.2×10^{-11} and 4.1×10^{-6} , respectively (Johnston, '15). To obtain more exact information concerning this behavior of Mg and Ca, the following experiments were run on sea water taken from the English Channel outside the Plymouth breakwater, and from Great Harbor, Woods Hole, Mass.

Graded amounts of 10 normal NaOH (practically carbonate-free²) were added to 100 c.c. portions of sea water. The flasks were stoppered and the contents thoroughly mixed. The supernatant fluid was filtered off as soon as the precipitate had settled somewhat (within four hours in all cases), and Ca and Mg were determined in separate samples of the filtrate. Ca was precipitated as oxalate from 25 cc. samples according to McCruden's ('09) method, and allowed to stand in the refrigerator for at least 18 hours. The oxalate, after washing, was determined with permanganate. The Mg determinations were carried out according to the method of Willstätter and Waldschmidt-Leitz ('23) on duplicate 5 cc. samples from each filtrate. Values for total Ca and Mg were obtained by the same techniques from samples of untreated sea water, and show good agreement with the figures compiled by Clarke ('24) for sea water from a wide range of sources.

¹ From the Laboratory of the Marine Biological Association, Plymouth.

² Made up from the filtrate of a 50 per cent. solution in which the carbonate had been allowed to settle.

The behavior of Mg and Ca was investigated by Irving¹ ('26), but major emphasis was placed by him on equilibria within the biological limits of alkalinity. An extension of these investigations and an explanation of certain discrepancies which were encountered follow.

The data for Mg for Plymouth sea water are given in Fig. 1, and roughly agree with my results obtained on Woods Hole sea water by a less reliable technique. The curve for the precipitation of Mg as drawn by Irving is inaccurate, as owing to the scarcity of his points he completely missed the plateau. Fig. 1, however, substantiates the points he did determine.

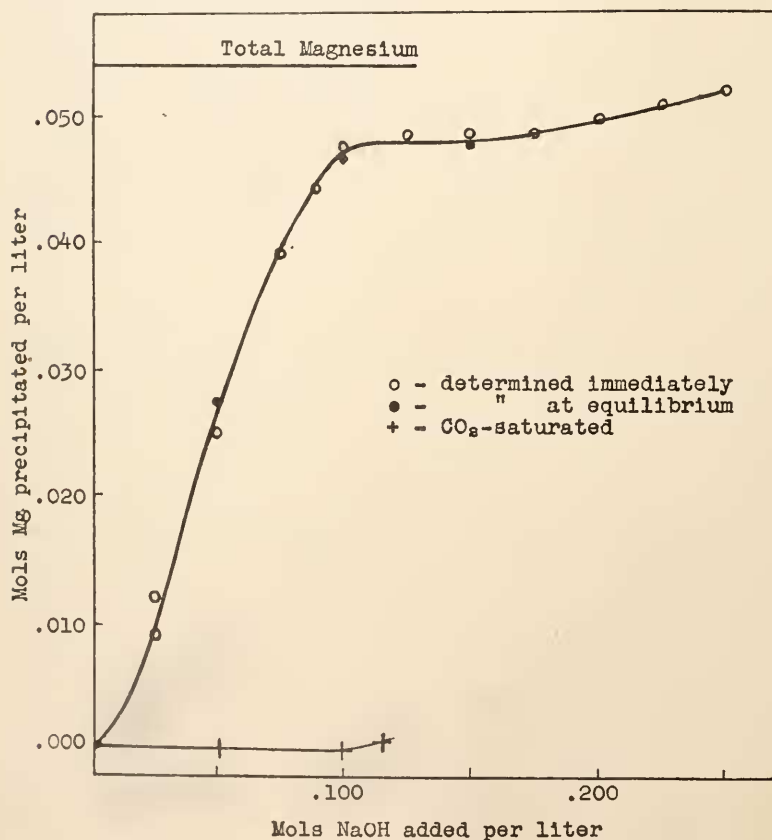


FIG. 1. The precipitation of Mg from Plymouth sea water in relation to the amount of NaOH added.

¹ Unknown to me when this work was undertaken.

The data for Ca show that the results may be considerably modified by a slight variation in procedure. The Ca curves plotted as hollow circles in Figs. 2 and 3 both differ markedly from the one obtained by Irving. His technique was substantially the same as mine, with the exception that his original samples of sea water, after the NaOH had been added, were shaken for 24 hours instead of being filtered at once, so that equilibrium was insured. Since CaCO_3 tends to remain supersaturated, it was suspected of being the cause of the discrepancy. A control experiment was therefore set up, in which the NaOH was added very slowly as a normal (instead of 10 normal) solution, in order to avoid local high concentrations of hydroxide, and the stoppered mixtures were allowed to stand with occasional shaking for one week. At the end of this time they were filtered and analyzed.

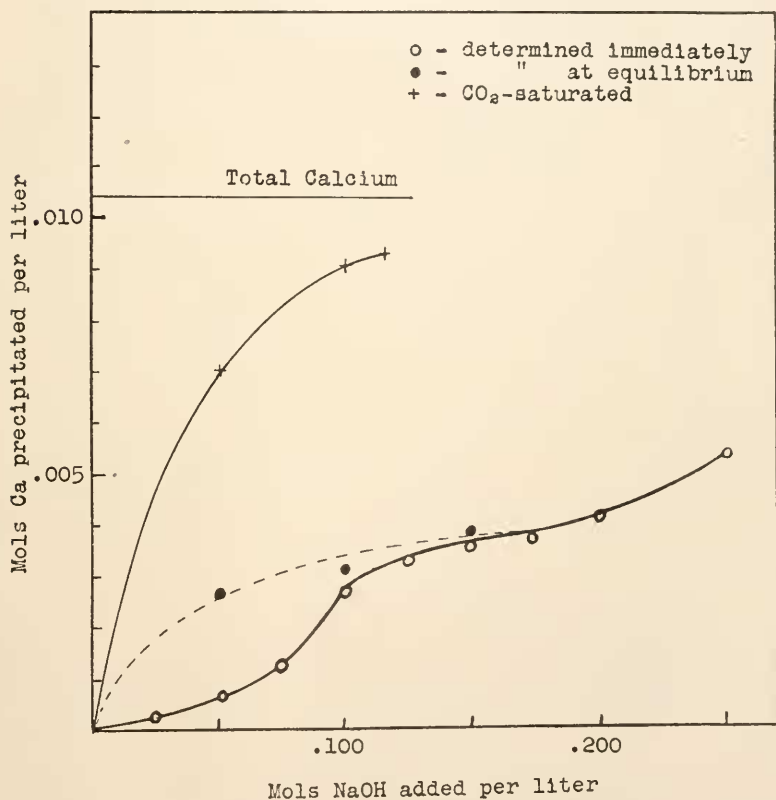


FIG. 2. The precipitation of Ca from Plymouth sea water in relation to the amount of NaOH added.

The Ca curve thus obtained differs from the first ones, this time confirming the results of Irving. Its points are shown in Fig. 2 as black circles. The difference between the two curves is therefore due only to the slowness with which CaCO_3 is precipitated, and can be controlled by taking the time factor into account. The same situation does not exist in the case of Mg, as can be seen from the black circles plotted in Fig. 1, which coincide with the original curve.

The effect of increasing the amount of carbonate was obtained by saturating several samples of sea water with CO_2 before the addition of the alkali. Increasing quantities of normal NaOH were then added very slowly, to allow the gelatinous precipitate which formed to redissolve, until the third sample, to which 11.5 cc. had been added, remained cloudy. The mixtures were aerated to drive off excess CO_2 , and allowed to stand in contact with the atmosphere for one week. During this time a crystalline precipi-

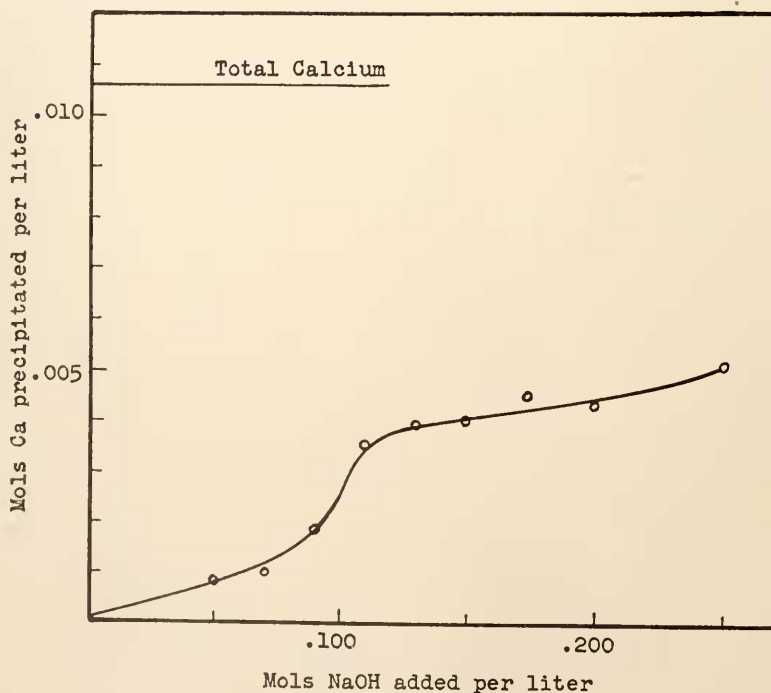


FIG. 3. The precipitation of Ca from Woods Hole sea water in relation to the amount of NaOH added.

tate had formed, and the solutions were filtered and analyzed as before. The results are shown by the crosses in Figs. 1 and 2, and are strikingly different from the other precipitations. In this case the addition of a small amount of alkali precipitates only the Ca, while the Mg is affected by larger amounts.

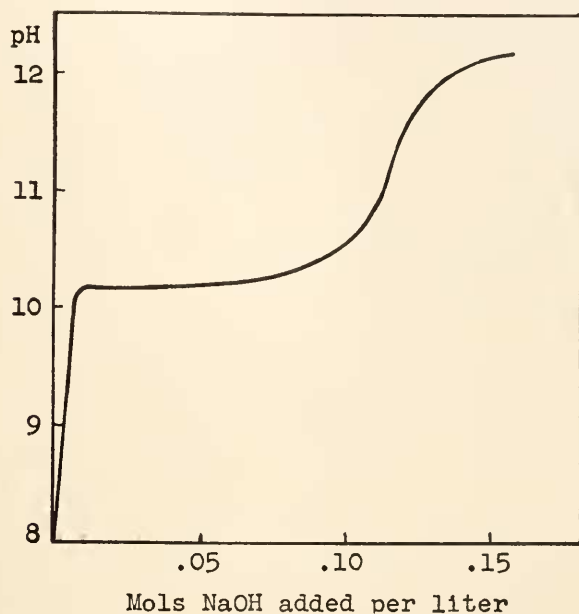


FIG. 4. The effect of NaOH on the pH of sea water (after Haas).

With reference to the reason for the shape of the Haas titration curve (Fig. 4), it is clear that Haas' own statement, mentioned previously, must be modified somewhat. As he suggested, Mg is precipitated rapidly by NaOH over the range where his titration curve shows a plateau. At a region corresponding to the addition of 0.1 mols of NaOH per liter of sea water, the titration curve begins its second rise, and the Mg curve flattens out. A small amount of Ca, however, is precipitated throughout, owing its first precipitation to the insolubility of the carbonate,¹ which is intermediate in this respect between Mg and Ca hydroxides.

I am deeply indebted to Dr. E. J. Allen, F. R. S., of the Marine Biological Association, Plymouth, for facilities extended to me

¹ $K_{S.P.} = .98 \times 10^{-8}$ (Johnston, '15).

during this investigation. I also wish to thank Prof. M. H. Jacobs and Mr. H. W. Harvey for their helpful interest.

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FURTHER OBSERVATIONS ON THE EFFECT OF HIGH FREQUENCY SOUND WAVES ON LIVING MATTER.

E. NEWTON HARVEY, ETHEL BROWNE HARVEY AND
ALFRED L. LOOMIS.¹

Interest in the biological effects of very high frequency sound waves started with the investigations of Wood and Loomis (1) who devised methods for producing intense "supersonic" vibrations and described many of the phenomena connected with them. The reader is referred to this paper for a description of the two kilowatt generator and methods of working with the waves. The apparatus was of such high power and the sound waves of such great intensity as to produce considerable heating. It seemed highly desirable in working with cells to reduce the heating effects of the vibrations, and to observe the cell with the microscope while being radiated. After many attempts to use the high power oscillator as the source of the waves and to lead them to the material on the stage of a microscope along capillary rods and tubes, a low-powered apparatus was decided upon as the most convenient for the purpose. This has previously been described by Harvey and Loomis (2) together with some of the effects of these supersonic waves on living organisms, cells and tissues. The outfit consists of a 75 watt high frequency oscillator and a quartz crystal whose vibrations, produced in the electric field by reversal of the piezo-electric effect, travel through any medium in contact with the crystal. A frequency of 400,000 per second was used and the material mounted directly on the crystal which served as a microscopic slide. Schmitt, Olson and Johnson (3) have also described various biological effects using a 250 watt generator with crystal immersed in xylene. They lead the sound waves along a rod of small diameter ending in a micro-needle, which could be inserted into the material to be studied.

Some additional effects have been recently observed with our

¹ From the Marine Biological Laboratory, Woods Hole, the Physiological Laboratory, Princeton University, and the Loomis Laboratory, Tuxedo Park, N. Y.

75 watt outfit in its original form and also modified to use higher frequencies by changing the capacity, inductance and crystal. The new quartz crystal was a spectacle lens which happened to be cut in the proper direction, kindly loaned by Dr. Kenneth Cole. The natural frequency of this crystal was approximately one and one quarter million per second. Its thickness varied from 1 to 1.8 mm. and consequently the distance between the tin foil electrodes, was much less than in the original 7 mm. crystal, giving a far more intense electrical field and greater effects. A few experiments have been made with a 2.25 million crystal which vibrates well and gives the same effects with *Elodea* as the 1.25 million. A 6 million crystal, 0.45 mm. thick, does not vibrate strongly. We are at present engaged in increasing the frequency to the highest point possible to see how biological effects will vary with the frequency.

A convenient means of finding the resonant frequency of the crystal is to set it up between the two tin foil electrodes with holes in their centers (to allow light to pass for microscopic observation) and then place a drop of water on the crystal. At various settings of the condenser the water will be violently agitated and broken up into fine droplets like steam. Low melting point crystals placed in the water show that the temperature does not rise but that the "steam" is mechanically formed, as observed in various ways by Wood and Loomis (1), and not a condensation from vapor. The exact specifications for an oscillator giving various frequencies will be found as an appendix to this paper.

If an *Elodea* leaf covered with a cover slip is mounted on a crystal whose resonant frequency is 400 kilocycles, and relatively weak (by reducing filament current) sound waves sent through the leaf, it can be observed under the microscope that only certain areas in the leaf show the characteristic whirling of the chloroplasts described in our previous paper (2). The areas do not correspond to any position on the crystal but to some peculiarity in the leaf, as moving a leaf to a new position over the crystal does not necessarily change the areas of marked whirling. These areas of whirling are most marked where air bubbles, which vibrate strongly, are caught under the leaf and where the cells are several layers in thickness, near the midrib (which also contains

air in intercellular spaces). Part at least of the condition for rapid whirling is the distance of the leaf from the crystal. By attaching the coverslip to a mechanical device for adjusting its distance from the crystal, the amount of water between coverslip and crystal can be varied and a slight change in this layer of water will cause whirling in a given area to start or to stop. These effects are no doubt due to interference of two sets of sound waves resulting in complicated interference patterns with nodes and internodes. Fine particles like red blood corpuscles suspended between crystal and coverslip can be observed to collect in nodes forming such a pattern. The chloroplasts in *Elodea* cells cannot do so since they are restricted in movement by the cell walls but in a region which happens to be an internode, they will undergo rapid whirling movements. The part played by an air bubble in causing rapid whirling is no doubt to offer a reflecting surface around which interference pattern and nodes appear. The whirling itself is probably due to the radiation pressure of the sound waves as they pass through the cells.

Another phenomenon regularly observed is a variation in the rate and character of the whirling as the variable condenser is changed to vary the frequency. For instance, over a range of 10 kilocycles, there appeared maximum whirling in a given area of the leaf at 407, 409, 410.4, 412.5, 415, and 417 kilocycles, *i.e.* a maximum approximately every 2 kilocycles, with no whirling or very slow whirling between.

In order to understand the changes in whirling motion imparted to the biological material placed upon the quartz as the frequency is varied, it is necessary to digress a moment and consider the forces acting upon an oscillating quartz disk. As is well known, a natural quartz crystal has three electric axes perpendicular to the optic axis. (See Fig. 1.)

The disk is cut as indicated by the shaded portion, *i.e.* so that one of the electric axes shall be perpendicular to the plane of the disk. If pressure is applied to the side of the disk corresponding to *A* — a negative charge will accumulate there, while correspondingly if a negative charge is applied there without pressure the disk will contract as if the equivalent pressure had been applied. The same holds true with positive charges on the *A* + side. On the other hand, when a positive charge is placed on the nega-

tive side and a negative charge on the positive side, the crystal will expand. A rapid alternation of charges causes the crystal to oscillate and as a first approximation the crystal can be considered to be an oscillating rigid piston. This would be rigorously correct if the disk were perfect and infinitely large but with a finite disk the forces are not symmetrical near the edges and a complex wave pattern is formed in the crystal. This can easily be seen by first considering a point O on the surface of the crystal near the center (Fig. 2). If a unit negative charge is placed on the under surface with the corresponding positive charge on the

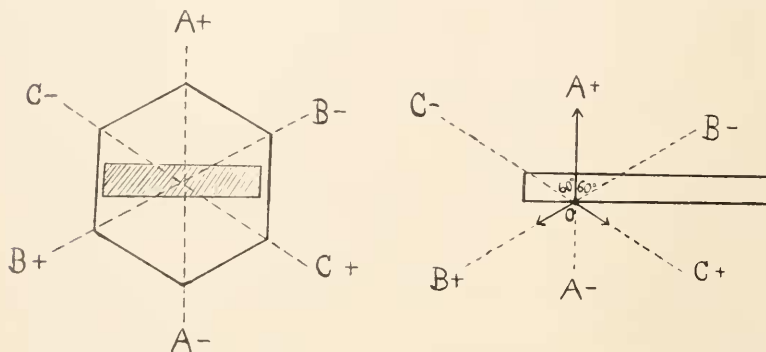


FIG. 1. Quartz crystal (shaded) cut perpendicular to optic axis. Electric axes indicated by AA , BB , CC .

FIG. 2. Vectors showing forces in point O in crystal.

upper, the crystal at " O " will tend to contract along the axis $OA+$ and expand along the axes $OB-$ and $OC-$. The intensities of these forces are directly proportional to the potential gradients along the respective axis. The forces along $OB-$ and $OC-$ are therefore only half as great as along $OA-$ since the distances through the crystal along these axis are twice as great as along the axis $OA-$ (the angles between the axis being 60°). The vector resolution of the forces $OC-$ and $OB-$ along $OA-$ shows that they are equivalent to a force opposed to the force along $OA-$ and of magnitude equal to one half that of $OA-$. The vector equivalent of all three forces is therefore a single force along $OA-$ equal to half of what that force would be if the forces along the axes $OC-$ and $OB-$ were not present.

This symmetry does not maintain however near the edges of the disk. Consider the point Q , Fig. 3. The axis QB is not in

the crystal at all. The resolution of the forces along $QA -$ and $QC -$ gives a force along QX equal to the force along $QA +$ multiplied by $\frac{1}{2}\sqrt{3}$. It is clear, therefore, that the forces near the edges are not symmetrical and tend to produce distortions which travel in waves across the disk.

A second system of forces are also acting on the disk. As the quartz contracts normally to the surface it expands parallel to the surface (this effect is best seen in a rectangular plate). Thus the series of longitudinal waves create interference patterns with the traverse waves.

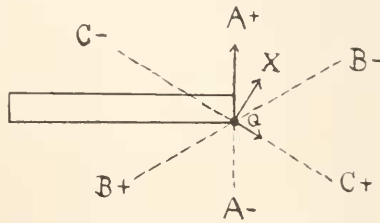


FIG. 3. Vectors showing forces in point Q in crystal.

Thirdly, it has been shown that even with a perfect quartz crystal the intensity of the piezo electric effect varies in different parts of the crystal. Dye has photographed the distortions produced in the interference fringes of an interferometer when one of the plates is an oscillating quartz disk. These photographs show most beautiful and complex patterns which slowly drift across the plate when the frequency is slowly changed.

Fortunately in biological investigations under the microscope good use can be made of these complex patterns. Thus, without changing the position of the specimen on the crystal, one can by merely changing slightly the frequency, cause these patterns to shift so that any particular part of the specimen can be made to experience forces of varying magnitude and direction. Thus in a particular cell of *Elodea* the chloroplasts can, at will, be made to rotate slowly or rapidly, clockwise, or counter clockwise, in one vortex or in a series of vortices, while merely watching the specimens under the microscope and observing the effects produced as the frequency is slowly varied.

By increasing the intensity, the leaf of *Elodea* can be agitated so violently that the chloroplasts themselves are broken up into a

fine green emulsion which completely fills the cell. This effect is not due to heating, since crystals of ethyl stearate, melting at 30–31° C. and placed on the *Elodea* leaf, are not melted even after 15 minutes, nor does slowly heating *Elodea* leaves bring about this effect. Neither is it due to possible mechanical rupture of the cellulose wall or mixing of the vacular sap with the chloroplasts, since unrayed cells can be crushed with a needle and their chloroplasts do not break up in this characteristic manner. The emulsification is caused by the tearing action of the sound waves.

Perhaps it should be emphasized at this point that these effects are all due to high frequency sound waves and not to any influence of the oscillating electrical field, as control experiments using glass plates of a size similar to and replacing that of the quartz crystal have shown.

Some of the more interesting of the effects observed with the spectacle lens crystal and frequencies of 1,250 kilocycles are as follows:

Amæba proteus or *dubia*,¹ moving along the surface of the crystal are not particularly affected by an intensity that causes the inclusions in small vacuoles of the *Amæba* to rotate on their axes. Higher intensities cause a mild whirling of the more liquid regions of the *Amæba* followed by rupture of the pellicle on one side and extrusion of the contents which join the general whirl of fluid in the medium. There is a tendency for the *Amæba* to move more rapidly during the raying as if the endoplasm became more liquid. After this there is a sudden change in direction of movement.

Both unfertilized and fertilized sea urchin (*Arbacia*) and starfish egg are violently agitated and may spin around. The jelly is torn off and the fertilization membrane may be broken. The eggs are thrown into rows or clumps and eventually cytolysis either partially or completely, the cytolysis taking place on one side and sometimes within the fertilization membrane. There is no movement of materials inside the egg caused by raying, as can be determined with certainty by using centrifuged eggs, the stratified layers remaining intact until cytolysis takes place. Cytolysis may take place from any of the stratified layers. However, if the unfertilized centrifuged eggs are placed in diluted sea water (40 distilled water to 60 sea water) and thus made less

viscous, the inside may be made to whirl. The whirling takes place in the lighter layers, including the oil, clear and granular layers, but only along the edge of the pigment layer, most of which remains intact. The oil drops tend to remain together, but the clear and granular layers become mixed and after ten or fifteen minutes of whirling a clear zone can not be distinguished. The direction of rotation may be reversed instantly by a slight change in frequency. Sometimes instead of a whirling of the protoplasm, there is a streaming of granules similar to that of an *Amœba*. No whirling of protoplasm nor movement of granules has been observed in eggs put in dilute sea water and not centrifuged. This may be due to the fact that the less dense material, where the whirling takes place is not separated out from the heavier pigment granules.

The asters are quite unaffected by raying. Cleavage furrows will come in normally during raying, even when the egg is violently agitated. When an egg has been slightly cytolyzed by raying we have observed that the furrow may come in at the proper place. Eggs in the two or four cell stage may have one or two blastomeres cytolyzed and the others unaffected.

Arbacia plutei swimming slowly are paralyzed by a momentary raying, presumably because the cilia are torn off. Otherwise they look uninjured but more prolonged treatment or greater intensity will tear them to pieces, leaving only fragments of the skeleton behind.

The gill cilia of *Mytilus* do not seem to be affected by violent agitation of the sea water about them, until the cilia and gill filaments are actually torn to pieces.

Pigment cells well-expanded in the scales of *Fundulus* are not affected, although the scales are rapidly agitated as the waves impinge upon them.

Frog abdominal muscles mounted on the crystal show no contraction or movement although air bubbles and blood corpuscles on top of the muscle tissue whirl rapidly. The waves must have passed through the muscle tissue to reach the air bubbles and corpuscles.

Fragments of the rays of the ctenophore, *Mnemiopsis*, containing luminous material, mounted on the crystal in the dark and waves passed through, are agitated and occasionally

luminesce. There is no continual luminescence which can be attributed to the waves but only the sporadic luminescence connected with sudden movement of the fragment such as can be obtained on jarring the table containing fragments of *Mnemiopsis*, even when not exposed to high frequency sound waves.

Fundulus embryos within the egg, with beating hearts, subjected to waves of an intensity to agitate the eggs but not so great an intensity as to interfere with observation of the heart beat show no marked effect upon the character of the beat or circulation. In fact only the effect observed was a slight increase in rate during raying which can be accounted for by a slight increase in temperature, that undoubtedly occurs when these high frequency waves carrying considerable energy, are absorbed by the medium. The embryos were rayed 1 minute and then not rayed for one minute while the heart beats were counted. In four experiments the rates were: Rayed—148, 157, 140, 132; unrayed—140, 148, 122, 122, respectively. The average increase in rate was about 8 per cent., which can be accounted for from the known effect of temperature on the heart beat of *Fundulus heteroclitus*,² by a rise of temperature from 22° C. to about 23° C.

Perhaps it should be emphasized again from the experiments on muscle, heart, luminous cells and chromatophores that there is no stimulating effect of these waves similar to the stimulation by electrical or sudden mechanical disturbance.

Fertilized *Fundulus* eggs mounted on the crystal can be very violently agitated and the oil drops and granules within made to dance. The yolk can be thoroughly stirred and the surface of the protoplasm can be observed to move and bend. Dr. Elmer Butler has carried these eggs to the point of hatching and finds the development and the embryos normal. If the agitation has continued so long as to burst the protoplasmic surface development does not proceed. An intensity of raying which does not destroy the surface has no effect on development while a slightly greater intensity results in dissolution and cytolysis.

Study of a large number of cells and tissues, some of which are recorded above, has led us to the conclusion that the effects of these waves, apart from slight heating, are purely mechanical. If intense enough, practically all cells can be cytolized. It is as

if one could grasp a cell in both hands and bend it violently back and forth at a very rapid rate. Delicate structures on the outside of a cell are torn off. If the cell is very small it is thrown into nodes so quickly as to escape injury. If the cell can be held fixed and is not too viscous, its contents can often be made to whirl before it breaks down.

From the whirling one can gain an idea of the viscosity of the cell contents. Perhaps the chief value of the waves for biological investigation lies in the evidence obtained from their action regarding the viscosity of cells. It should be emphasized, however, that comparative studies of viscosity are difficult because of the great complexity of the sound wave patterns under the cover slip, both horizontally and vertically. Two cells in different portions of the same microscopic field are not necessarily exposed to the same radiational forces and great caution must be used in drawing conclusions regarding viscosity or resistance to tearing by difference in behavior of cells.

High frequency sound waves offer a new means of affecting the interior of cells without necessarily breaking down the cell wall. They will be of most value when a beam of given frequency and controlled intensity can be sent through a cell or tissue in a particular direction.

APPENDIX.

For those biologists who desire to construct a low-powered oscillator, the following constructional details ought to suffice.

The following apparatus is recommended.

One No. 852 Radiotron 75 watt tube,

One tube holder

One filament transformer to give 10 volts

One plate transformer to give 2,000 volts

One 5,000 ohm resistance

Several transmitting condensers (designed to withstand 5,000 volts) with an aggregate capacity of about 0.1 microfarad

One rheostat

Some heavy copper strip to wind the inductance

Some fine wire to make the secondary

All of the above can be bought from any radio store carrying

parts for transmitting sets, and should not cost more than \$100 in the aggregate.

Fig. 4 shows the wiring diagram and a suggested arrangement of the parts. The iron of the transformers should be on the side of the tube away from the oscillating parts and should be at least a foot from the tube. All the parts can conveniently be mounted on a board 30 x 10 inches.

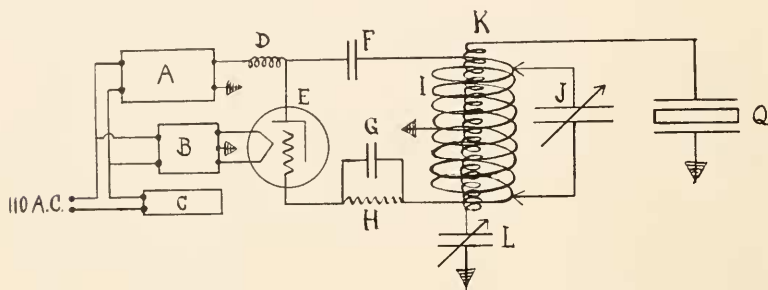


FIG. 4. Constructional diagram for a 75 watt oscillator. A, plate transformer; B, filament transformer; C, rheostat; D, choke coil; E, Radiotron No. 852; F, Blocking condenser. G, Grid leak condenser. H, Grid lead. I, Inductance coil; J, tuning condenser; K, secondary coil; L, Variable condenser; Q, Quartz plate between electrodes.

The rheostat should be mounted in the lead from the 110-volt A. C. house circuit and can be used to regulate the voltage. The primaries of the transformers should be connected in parallel across the house circuit. One side of the secondary of the plate transformer should be connected to the center tap of the filament transformer which point should also be grounded. The other side of the secondary should go through a choke coil to the plate. The choke coil can be made by winding about 100 turns of fine wire on a bakelite tube one or two inches in diameter.

The inductance can be made by winding fifteen or twenty turns of heavy copper wire on a bakelite tube six or eight inches in diameter. The plate should be connected to one tap on the inductance through a blocking condenser of about .002 microfarad capacity. The grid should be connected to the other tap on the inductance through a by-pass condenser of about the same capacity and a grid leak of above 5,000 ohms resistance. The center tap of the inductance should be grounded. The secondary can be made by winding 100 turns of fine wire on a bakelite tube

which can be slipped inside the primary inductance. One end of the secondary should go to one plate of the crystal holder (the other plate of the holder being grounded). The other end of the secondary should be connected to ground through a variable condenser or to a rod of metal perhaps 1 inch diameter and ten inches long, which is not grounded.

The quartz crystal need not be larger than one square inch. It should be cut perpendicular to an electric axis. Its natural frequency of oscillation will depend on its thickness.

Mm. Thick.	Frequency (Approx.).
1.....	2,900,000 cycles per sec.
2.....	1,450,000 " " "
3.....	966,000 " " "
4.....	725,000 " " "
5.....	580,000 " " "
etc.....	etc.

The oscillating circuit should be tuned to approximately the frequency of the crystal.

The crystal holder can conveniently be made out of two microscope slides and two thin brass strips with holes cut in them for use with the microscope. The microscope should be at least three feet from the oscillator so that movements of the operators body shall not change the frequency. The high tension lead to the microscope should be shielded by surrounding it with a grounded metal tube and the microscope itself should be grounded to prevent small spark discharges to the observer.

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¹ Kindly supplied by Dr. J. A. Dawson of Harvard University.

² Unpublished data of Dr. Otto Glaser of Amherst College.

BIOLOGICAL BULLETIN

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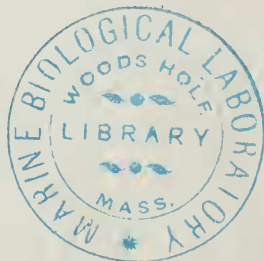
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